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(57) Abstract

The present invention relates to the epitopes of hepatitis C virus (HCV) core protein and non-structural 3 protein (NS3), and the epitopes of envelope protein, epitopes of non-structural 4 protein, and the epitopes of HCV non-structural 5 protein and a recombinant protein comprising the same; processes for producing the recombinant proteins; an agent for diagnosing antibodies against hepatitis C virus in a putative serum sample, which comprises said recombinant proteins; and a process for diagnosing hepatitis C by using the agent.

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IMPROVED HCV DIAGNOSTIC AGENTS

FIELD OF THE INVENTION

The present invention relates to hepatitis C virus(HCV)-specific epitopes, recombinant proteins comprising one or more of the HCV epitopes and a process for detecting antibodies against hepatitis C virus in a putative serum sample by using said recombinant proteins. More particularly, it pertains to epitopes of HCV core protein and non-structural 3 protein(NS3), and a recombinant protein comprising the same, epitopes of HCV envelope protein and Non-structural 4 protein, and a recombinant protein comprising the same, and epitopes of HCV non-structural 5 protein and a recombinant protein comprising the same; processes for producing the recombinant proteins; an agent for diagnosing antibodies against hepatitis C virus in a putative serum sample, which comprises said recombinant proteins; and a process for diagnosing hepatitis C by using the agent.

BACKGROUND OF THE INVENTION

Hepatitis C virus(HCV) is a primary cause of viral hepatitis which progresses into cirrhosis or hepatocellular carcinoma, and it has been reported that about 70 to 80% of hepatitis caused by blood transfusion is due to said virus(Alter, H. J., et al., Lancet, 2, 838-841(1975); and Dienstag, J. L., et al., Seminar Liver Dis., 6, 67-81(1986)). Said virus is one of RNA viruses consisting of one positive RNA strand and produces a polyprotein precursor from an open reading frame(ORF) of the strand(Choo, Q. L., et al., Science, 244, 359-362(1989); and, Choo, Q. L., et al., Proc. Natl. Acad. Sci. USA, 88, 2451-2455(1991)).

The gene structure of hepatitis C virus is similar to that of flavivirus or pestivirus(Miller, R. H., et al., Proc. Natl. Acad. Sci. USA, 87, 2057-2061(1990); and, Muraiso, K., et al., Biochem. Biophys. Res. Commun., 172, 511-516(1991)),

and on the basis of said relationship it is presumed that the polyprotein of hepatitis C virus consists of, from N-terminal to C-terminal, core-envelope 1(E1)-envelope 2/non-structural 1 protein(E2/NS1)-non-structural 2 protein(NS2)-non-structural 3 protein(NS3)-non-structural 4 protein(NS4)-non-structural 5 protein(NS5)(Choo, Q. L., et al., Proc. Natl. Acad. Sci. USA, 88, 2451-2455(1991); Takamizawa, A., et al., J. Virol., 65, 1105-1113(1991); and Kato, N., et al., Proc. Natl. Acad. Sci. USA, 87, 6524-6528(1990)).

The infection of hepatitis C virus can be diagnosed by detecting hepatitis C viral RNA directly from a blood sample by using polymerase chain reaction(PCR)(Hosoda, K., et al., Hepatology, 15, 777-781(1992); Abe, K., et al., Hepatology, 15, 690-695(1992); and, Alter, H. J., Annals of Internal Medicine, 115, 644-649(1991), whereby the viral RNA can be detected rather early, i.e., within 1 to 2 weeks from the infection; however, such method entails high cost and long time due to the need to analyze numerous samples. Another diagnostic method is to detect antibodies against hepatitis C virus present in the serum sample, e.g., by an enzyme-linked immunoassay using C100-3 protein(see Houghton et al., PCT WO 89/04669; WO 90/11089). Kuo et al. disclosed in Science 244, 362-384(1989) that more than 70% of patients with post-transfusion hepatitis have antibodies against the C100-3 protein.

However, said C100-3 antigen used as an active ingredient for the diagnostic agents reacts only to the antibodies of patients with chronic hepatitis C, not with those of patients with acute hepatitis C at its early stage since the antibodies are not generally produced until 4 to 6 months after the HCV infection. As a result, it often exhibits a false negative during the early stage of the disease(Alber, H. J., et al., N. Engl. J. Med., 321, 1494-1500(1989); Myamura, T., et al., Proc. Natl. Acad. Sci. USA, 87, 983-987(1990)); and, further, it often exhibits false positive results in a considerable proportion in the case of hepatitis caused by the autoimmune disease of the patients and not by HCV(McFarlane, I. G., et al., Lancet, 335, 754-

757(1990)).

Okamoto et al. disclosed the nucleotide sequences of the cDNA clones including the 5'-terminal region and structural genes encoding the core protein and envelope protein by using the HCV taken from the serum collected from Japanese hepatitis C patients, and compared said sequences with those of HCV extracted from the serum of chimpanzee which was prepared by Chiron Co. in the U. S. From that result, Okamoto et al. discovered the existence of a subspecific hepatitis C virus and the specificity of the antigens derived from Japanese type HCV for preparing vaccines and diagnostic agents against Japanese type HCV(Jpn. J. Exp. Med., 60, 167-177(1990)). Harada et al. further reported in J. Virol., 65, 3015(1991) that, when the core protein encoded in 5'-terminal portion of the structural gene was used as an antigen for diagnosing anti-HCV antibodies which may be present in the samples taken from putative patients, the antibodies could be detected 6 to 8 weeks earlier than the case of using C100-3 protein.

Further, Choo et al. disclosed an improved diagnostic method using a core protein expressed from a core structural gene and C33C protein expressed from NS3 gene(Br. Med. Bull., 46, 423-441(1990)); and, Okamoto et al. employed synthetic polypeptides synthesized by using the nucleotide sequences of a part of core structural gene to diagnose hepatitis C. UBI Co. of the U.S. developed another diagnostic method wherein synthetic polypeptides consisting of 15 to 65 amino acids encoded in core structural gene were employed as antigens for detecting anti-HCV antibodies(Wang, C.Y., EP 442394(1991); Hosein, B., et al., Proc. Natl. Acad. Sci., 88, 3647-3651(1991). In addition, Ortho Diagnostic Systems Inc. of the U. S. reported a second generation diagnostic agent having improved sensitivity for anti-HCV antibodies which was prepared by adding core antigen C22-3, NS3 partial protein C33C and NS4 partial protein C200 to the pre-existing first generation diagnostic agent(McHutchison, J. G., et al., Hepatology, 15, 19-25(1992)); and Alter describes that it it is possible to detect anti-HCV antibodies from a serum taken

from an HCV patient 15 to 20 weeks after the infection by HCV (Annals of Internal Medicine, 115, 644-649(1991)).

The present inventors also disclosed an intrinsic gene structure of Korean type hepatitis C virus(KHCV) different from the American type or Japanese type HCVs; expressed KHCV UBCORE14 protein from KHCV CORE gene, KHCV UB897 protein from KHCV NS3 gene, KHCV 403 protein from KHCV NS5 gene and envelope proteins from KHCV envelope gene in recombinant yeast or E. coli cells; confirmed the immunospecificity of the above expressed proteins; reported the process for purifying said proteins; and developed an improved diagnostic method to detect anti-HCV antibodies from a serum taken from a hepatitis C patient with the KHCV antigenic proteins by employing enzyme-linked immunosorbent assay(ELISA) (see Korean Patent Publication No. 93-683).

The present inventors have endeavored to develop HCV diagnostic agents with an improved accuracy and speed over the above agents. As a result, there have been unexpectedly discovered several HCV epitopes which react with the antibodies against HCV with a greater sensitivity and accuracy.

SUMMARY OF THE INVENTION

Accordingly, it is a primary object of the present invention to provide said epitopes of improved reactivity with HCV antibodies; and recombinant proteins comprising one or more HCV epitopes.

Another object of the present invention is to provide nucleotide sequences encoding said epitopes or said recombinant proteins; recombinant expression vectors comprising a nucleotide sequence encoding said recombinant protein which can produce, upon its expression, the recombinant protein comprising one or more HCV epitopes; and a host cell transformed with the recombinant expression vector.

An additional object of the present invention is to provide a process for producing a recombinant protein

comprising one or more HCV epitopes, which comprises culturing said host cell transformed with the recombinant expression vector containing a nucleotide sequence encoding said recombinant protein.

A further object of the present invention is to provide a diagnostic agent comprising one or more recombinant proteins which contain one or more HCV epitopes as (an) active component(s) for detecting anti-HCV antibodies in a putative sample; and a diagnostic kit comprising said agent.

A still further object of the present invention is to provide a process for diagnosing HCV infection at its early stage with speed and accuracy by employing said agent or kit.

In accordance with one aspect of the present invention, there are provided HCV epitopes comprising: KHCV NS4E, an epitope of HCV non-structural 4 protein; KHCV E1G, KHCV E2A and KHCV E2E proteins, epitopes of HCV envelope protein; KHCV COREEPI protein, an epitope of HCV core protein; KHCV 518 protein, an epitope of HCV non-structural 3 protein; and, KHCV NS5-1,2 protein comprising an epitope of non-structural 5 protein; and recombinant proteins comprising one or more said epitopes.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of preferred embodiments taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the nucleotide sequences encoding the epitope of core protein(COREEPI) and the epitope of non-structural 3 protein; and the amino acid sequences of the polypeptides encoded therein, respectively;

Fig. 2 depicts the nucleotide sequences encoding the epitope of NS4 protein(NS4E) and the epitopes of nonstructural 3 protein(E1G, E2A and E2E); and the amino acid sequences of the polypeptides encoded therein, respectively;

Fig. 3 describes the nucleotide sequence encoding KHCV NS5-1.2 protein and the amino acid sequence of the

polypeptide encoded therein;

Fig. 4 represents the position of each of the primers used for amplifying the KHCV897 DNA fragment by polymerase chain reaction(PCR);

Fig. 5 describes an expression vector constructed for the purpose of expressing a KHCV897 DNA fragment in Escherichia coli cells;

Fig. 6 A represents the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of a KHCV897 DNA fragment in E. coli cells, and Fig. 6 B shows the result of western blotting analysis with the gel of Fig. 6 A by using a serum taken from a hepatitis C patient;

Fig. 7 represents the position of each of the primers used for amplifying various DNA fragments of a gene encoding an envelope protein by PCR;

Fig. 8 portrays an expression vector constructed for the purpose of expressing a DNA fragment encoding a portion of an envelope protein in E. coli cells;

Fig. 9 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of various DNA fragments encoding a portion of an envelope protein in E. coli cells, and Fig. 9 B shows the result of western blotting analysis with the gel of Fig. 9 A by using a serum taken from a hepatitis C patient;

Fig. 10 delineates a schematic diagram for preparing an expression vector constructed for the purpose of expressing UBCORE518 protein;

Fig. 11 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of UBCORE518 DNA fragment in E. coli cells, and Fig. 11 B shows the result of western blotting analysis with the gel of Fig. 11 A by using a serum taken from a hepatitis C patient;

Fig. 12 shows a schematic diagram for preparing an expression vector constructed for the purpose of expressing a recombinant DNA comprising a ubiquitin gene and the DNA fragment encoding an epitope of non-structural protein 4(NS4E protein);

Fig. 13 shows a schematic diagram for preparing an

expression vector constructed for the purpose of expressing a recombinant DNA encoding UBE1E2 protein comprising ubiquitin and the fusion protein E1E2 containing epitopes of envelope protein, E1G, E2A and E2E;

Fig. 14 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of recombinant UBE1E2 protein in E. coli cells;

Fig. 14 B shows the result of western blotting analysis with the gel of Fig. 14 A by using a serum taken from a hepatitis C patient;

Fig. 15 shows a schematic diagram for preparing an expression vector constructed for the purpose of expressing a recombinant DNA encoding UBNS4E1E2 protein comprising ubiquitin, NS4E protein and E1E2 protein;

Fig. 16 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of the recombinant DNA encoding UBNS4E1E2 protein in E. coli cells;

Fig. 16 B shows the result of western blotting analysis of the gel of Fig. 16 A by using a serum taken from a hepatitis C patient;

Fig. 17 shows a schematic diagram for preparing an expression vector constructed for the purpose of expressing a recombinant DNA encoding USNS5-1.2 protein comprising ubiquitin and NS5-1.2 protein;

Fig. 18 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of the recombinant DNA encoding UBNS5-1.2 protein in E. coli cells, and Fig. 18 B shows the result of western blotting analysis with the gel of Fig. 18 A by using a serum taken from a hepatitis C patient; and

Fig. 19 A shows the result of SDS polyacrylamide gel electrophoresis(SDS-PAGE) after the expression of KHCV403 protein and recombinant UBNS5-1.2 protein in E. coli cells, and Fig. 19 B shows the result of western blotting analysis with the gel of Fig. 19 A by using a serum taken from a hepatitis C patient.

DETAILED DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, the following terms shall have the following meanings:

The term "hepatitis C virus" refers to a virus causative of non-A non-B hepatitis or hepatitis C. The terms HCV and hepatitis C are used interchangeably herein.

The term "Korean-type hepatitis C virus" or "KHCV" refers to a novel type of HCV which is isolated from Korean hepatitis C patients; and whose cDNA has an open reading frame of a nucleotide sequence encoding the amino acid sequence, wherein the amino acids having the numbers of 842, 849 and 853 are phenylalanine, leucine and threonine; or leucine, phenylalanine and alanine, respectively.

The term "epitope" refers to an antigenic determinant of a polypeptide which is capable of eliciting an immune response in an immunologically competent host organism and/or is capable of specifically binding itself to a complementary antibody. An epitope in accordance with the present invention generally consists of at least 6 amino acids, preferably 7 or 8 amino acids.

Other terms used herein have the normal and conventional meanings as practiced and understood in the art.

Hereinafter, the number of a nucleic acid of an HCV cDNA or of an amino acid of an HCV protein is based on the full KHCV nucleotide sequence or amino acid sequence disclosed in Korean Patent Laid-open Publication No. 93-683.

The present invention will now be more specifically illustrated hereinbelow.

1. Determination of Epitopes

The information on nucleotide sequences of cDNAs of HCV, for example, KHCV(KHCV-LBC1, which was deposited at American Type Culture Collection(ATCC) on May 14, 1991 with the accession number of ATCC 75008; Korean Patent Laid-open

corresponding to from the 2281st to the 2529th nucleotides of KHCV cDNA(E2E protein)(see Fig. 2 for the amino acid and nucleotide sequences of E1G, E2A and E2E proteins).

In addition, epitopes of NS5 protein exists in the amino terminal of NS5 protein encoded by 1,200 base pairs corresponding to from 6649th to 7824th nucleotides including KHCV403 cDNA fragment and reacts specifically with a serum taken from a KHCV patient with a higher sensitivity than KHCV403.

2. Recombinant Proteins Comprising One or More HCV Epitopes

Epitopes of HCV antigens are very important for the development of efficient and economical diagnostic agents and vaccines. In particular, the fusion proteins comprising one or more epitopes are more preferable in terms of economy, efficiency and accuracy; and the fusion proteins comprising more than one epitope are most preferable.

As a HCV recombinant protein comprising more than one HCV epitope, there may be included, preferably, a recombinant CORE 518 fusion protein comprising the epitopes of KHCV core and NS3 proteins, and a recombinant NS4E1E2 fusion protein comprising the epitopes of KHCV E1, E2 and NS4 proteins.

The recombinant proteins may be prepared by employing various expression vector systems containing a nucleotide sequence encoding said fusion protein; and, the vector may be capable of directing production of a recombinant fusion protein comprising said fusion protein and other specific protein, preferably, ubiquitin which can increase the protein stability or facilitate the purification procedure.

For instance, a desired HCV protein can be obtained by expressing a fused polynucleotide of a HCV cDNA fragment and ubiquitin gene in bacteria such as Escherichia coli, and then excising the ubiquitin in vitro by a ubiquitinase named UBP 1 (Tobias, J. W. et al., J. Biol. Chem., 266, 12021-12028 (1991)). The recombinant fusion protein comprising ubiquitin as well as the KHCV fusion protein can be used in accordance with the invention as long as it retains the necessary

characteristic of KHCV protein, e.g., antigenicity of HCV.

The above expression system may be effectively employed where the desired protein is unstable and can be digested easily by proteinases in a host cell since the ubiquitin can protect the desired protein from the protease attack or stabilize it. Moreover, the expression of desired recombinant protein fused with ubiquitin can be confirmed by using anti-ubiquitin antibodies and easily purified by using the properties of ubiquitin.

For the purpose of obtaining a desired HCV protein comprising HCV epitopes, a compatible host cell is transformed with an expression vector containing an HCV cDNA fragment encoding HCV epitopes; and the transformed cell is cultured under a condition that allows the expression.

Selection of an appropriate host organism is affected by a number of factors as well known in the art. These factors include, for example, compatibility with the chosen vector, toxicity of the proteins encoded by the recombinant plasmid, ease of recovery of the desired protein, protein characteristics, biosafety and costs. A balance of these factors must be considered, it is being understood that not all hosts will be equally effective for the expression of a particular recombinant DNA molecule.

Suitable host organisms which may be used in the invention include, but are not limited to, bacteria such as Escherichia coli and yeasts such as Saccharomyces cerevisiae.

The polypeptides produced in a host cell may be isolated and purified by a combined use of conventional methods, e.g., cell disruption, centrifugation, dialysis, salting-out, chromatography, gel filtration, electrophoresis and electroelution.

The polypeptides of the invention can also be chemically synthesized by a suitable method such as exclusive solid phase synthesis, partial solid phase method, fragment condensation or classical solution synthesis. The method of solid phase synthesis disclosed by Merrifield(J. Am. Chem. Soc., 85, 2149(1963)) is preferred.

On the other hand, amino acid substitutions in proteins

which do not substantially alter biological and immunological activities have been known to occur and have been described, e.g., by Neurath et al., in The Proteins, Academic Press, New York(1979), in particular in Fig. 5 appearing on page 14 thereof. Such functionally equivalent amino acid substitutions are believed to fall within the scope of the invention as long as the resulting proteins retain the same antigenic properties.

In this specification, standard three-letter abbreviations are used to represent nucleotides and amino acids. The meanings of these abbreviations can be found in standard biochemistry textbook, e.g., Lehninger, Principles of Biochemistry, Worth Publishers Inc., New York, pp. 96, 798(1984).

1) Preparation of CORE518 protein

The information on nucleotide sequences of cDNAs of Korean type hepatitis C virus(see Korean Patent Laid-open Publication No. 93-683) was used to synthesize primers for polymerase chain reaction which correspond to the 5'- and the 3'-ends of KHCV518 cDNA fragment encoding KHCV518 protein, which comprises the epitope of KHCV NS3 protein. A primer corresponding to the 5'-end of cDNA is designed to have recognition site of endonuclease in its the 5'-end so as to ligate with the 3'-end of COREEPI gene encoding epitope of core protein, and a primer corresponding to the 3'-end of cDNA is designed to have a termination codon and recognition site of endonuclease. Therefore, said primers allow the synthesis of fusion protein to start from the initiation codon of ubiquitin and then end at the inserted termination codon, and facilitates the cloning of the fused gene into the expression vector.

Moreover, it is possible to arrange said genes encoding said two epitopes in an opposite order by regulating the sequence of the primers properly, and it is also possible to insert other amino acid sequences between the two epitopes as long as the resulting proteins retain the same antigenic properties.

A polymerase chain reaction is carried out by using said primers and KHCV897 gene(which was deposited at ATCC on June 27, 1991 with the accession number of ATCC 68640) as a template to amplify KHCV518 gene, and the gene fragment obtained by digesting said KHCV518 gene with restriction endonucleases is inserted into a plasmid ptrp-UB-CORE14 in place of part of KHCV Core14 gene exclusive of COREEPI gene to obtain an expression vector thereof. The expression vector is used to transform suitable host organisms such as E. coli. The polypeptides produced by the transformed host cell are subjected to an electrophoresis on 15% polyacrylamide gel and then to a western blotting analysis by using a serum taken from a hepatitis C patient to confirm that the CORE518 protein reacts specifically with anti-KHCV antibodies.

2) Preparation of NS4E1E2 protein

The information on nucleotide sequences of cDNAs of Korean type hepatitis C virus(see Korean Patent Laid-open Publication No. 93-683) is used to ligate the nucleotide sequences encoding the epitope of NS4 protein(NS4E) and the epitopes of envelope proteins(E1G, E2A and E2E), for example, by using polymerase chain reaction with appropriate primers. First of all, primers for PCR which correspond to the 5'- and 3'-ends of nucleotide sequence encoding E1E2 protein, which comprises the epitopes of envelope proteins, i.e., E1G, E2A and E2E . A primer corresponding to the 5'-end of said nucleotide sequence is designed to have 21 nucleotides the same as those in the 3'-end of NS4 gene in its the 5'-end so as to ligate with the 3'-end of NS4 gene, and a primer corresponding to the 3'-end of cDNA is designed to have a termination codon and recognition site of endonuclease. Therefore, said primers allow the synthesis of fusion protein to start from the initiation codon of ubiquitin and then end at the inserted termination codon, and facilitates the cloning of the fused gene into the expression vector.

Moreover, it is possible to arrange said four genes encoding the respective epitopes in an optional order by regulating the sequence of the primers properly, and it is

also possible to insert other amino acid sequences between any two epitopes as long as the resulting proteins retain the same antigenic properties.

A polymerase chain reaction is carried out by using said primers and plasmid ptrpH-UB-E1E2(see Fig. 16) as a template to amplify E1E2 gene. A second polymerase chain reaction is carried out by using E1E2 and NS4 gene as templates to amplify NS4E1E2 gene, and the gene fragment obtained by digesting said NS4E1E2 gene with restriction endonucleases is inserted into a plasmid ptrp-UB-CORE14 in place of KHCV Core14 gene to obtain an expression vector thereof. The expression vector is used to transform suitable host organisms such as E. coli W3110(ATCC 37339), and the transformed host cell is cultured under a condition that allows the expression. The polypeptides produced by the transformed host cell are subjected to electrophoresis on 15% polyacrylamide gel and then to a western blotting analysis by using a serum taken from a hepatitis C patient to confirm that the NS4E1E2 protein reacts specifically with anti-KHCV antibodies.

3) Preparation of NS5-1.2 protein

The information on nucleotide sequences of cDNAs of Korean type hepatitis C virus(see Korean Patent Laid-open Publication No. 93-683) is used to synthesize primers for polymerase chain reaction which correspond to the 5'- and 3'-ends of NS5-1.2 cDNA fragment encoding NS5-1.2 protein. A primer corresponding to the 5'-end of cDNA is designed to have a recognition site of endonuclease in its the 5'-end so as to ligate with the 3'-end of ubiquitin gene, and a primer corresponding to the 3'-end of cDNA is designed to have termination codon and recognition site of endonuclease. Therefore, said primers allow the synthesis of fusion protein to start from the initiation codon of ubiquitin and then end at the finished by inserted termination codon, and facilitates the cloning of the fused gene into the expression vector.

A polymerase chain reaction is carried out by using said

primers and KHCV-LBC1 gene(which was deposited at ATCC on May 14, 1991 with the accession number of ATCC 75008; see Korean Patent Laid-open Publication No. 93-683) as a template to amplify NS5-1.2 gene, and the gene fragment obtained by digesting said NS5-1.2 gene with restriction endonucleases is inserted into a plasmid ptrp-UB-CORE14 in place of KHCV Core14 gene to obtain an expression vector thereof. The expression vector is used to transform suitable host organisms such as E. coli. The polypeptides produced by the transformed host cell are subjected to an electrophoresis on 15% polyacrylamide gel and then to a western blotting analysis by using a serum taken from a hepatitis C patient to confirm that the NS5-1.2 protein reacts specifically with anti-KHCV antibodies.

3. Preparation of Diagnostic agent for hepatitis C comprising mixed HCV antigen polypeptides

The diagnostic agent in accordance with the present invention comprises one or more HCV epitopes including KHCV NS 4E, KHCV E1G, KHCV E2A, KHCV E2E, COREEPI, KHCV 518 and KHCV NS5-1.2, and/or recombinant proteins comprising one or more of said epitopes.

Further, the present invention provides a hepatitis C diagnostic kit which comprises the necessary agents to carry out the above procedure, essentially consisting of a diagnostic agent containing KHCV polypeptide(s) which carries one or more KHCV epitopes.

Preferably, it comprises a recombinant CORE518 fusion protein comprising the epitopes of KHCV core and NS3 proteins, a recombinant NS4E1E2 fusion protein comprising the epitopes of KHCV E1, E2 and NS4 proteins, and/or a recombinant NS5-1.2 protein.

When the diagnostic agent comprises more than one recombinant protein comprising HCV epitope(s) in a mixture, the proportion of each protein may be optionally adjusted, although it is generally preferable to use each protein in an equal molar amount.

The novel diagnostic method in accordance with the present invention comprises the following steps:

(i) a diagnostic agent containing one or more KHCV polypeptides is added to a solid support, e.g., a well of microtiter plate to make said KHCV antigen adsorb onto the surface of the well;

(ii) a putative sample diluted with a diluent is added to the antigen-coated well where the antigen-antibody complex is to be formed should there be any anti-KHCV antibodies in the serum;

(iii) an enzyme, e.g., HRP(horseradish peroxidase) conjugated anti-human IgG antibody is added to the well to allow the anti-human IgG antibody-HRP to bind the antibodies of the complex formed in step(ii); and

(iv) substrates for the enzyme, e.g., O-phenylene diamine dihydrochloric acid(OPD) and hydrogen peroxide for peroxidase are added to the well to develop a color reaction. When the putative serum contains anti-KHCV antibodies, color appears as a result of the reaction of the enzyme with the substrates. The color reaction is stopped by an addition of diluted sulfuric acid.

The degree of color intensity can be measured with a microwell reader; and the existence of anti-HCV antibodies can be determined on the basis of the result. The solid support for the diagnostic method may be of polystyrene beads or nitrocellulose strips.

In case that the recombinant protein(s) of the present invention which comprises more than one KHCV epitope is used for preparing a diagnostic agent, it would allow a more economical and accurate diagnosis than a case using any of the existing HCV antigens with only one epitope in a mixture. Moreover, the diagnostic agent and diagnostic kit of the present invention which comprise a mixture of recombinant proteins comprising KHCV epitopes show an excellent diagnostic result.

The following Examples are intended to further illustrate the present invention without limiting its scope; and the experimental methods used in Examples are practiced

in accordance with Reference Examples given hereinbelow, unless otherwise stated.

Further, percentages given below for solids in solid mixtures, liquids in liquids and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically defined otherwise.

Reference Example 1: Digestion of DNA with Restriction Endonuclease

In a sterilized 1.5mℓ eppendorf tube were added restriction endonuclease and reaction buffers to be a reaction volume ranging from 50 to 100μℓ, and the reaction was carried out at a temperature of 37°C for 1 to 2 hours. When the reaction was completed, the reaction mixture was heat-treated at 65°C for 15 minutes(or extracted with phenol and precipitated with ethanol in the case of a heat-resistant endonuclease) to inactivate the restriction endonuclease.

Restriction enzymes and reaction buffers used in this example were purchased from NEB(New England Biolabs, Jolla, MA, U.S.A.).

10 x reaction buffer for the reaction of a restriction endonuclease has the following composition:

10 x NEB reaction buffer 1: 100mM bis Tris propane-HCl, 100mM $MgCl_2$, 10mM dithiothreitol(DTT), pH 7.0

10 x NEB reaction buffer 2: 100mM Tris-HCl, 100mM $MgCl_2$, 500mM NaCl, 10mM DTT, pH 7.0

10 x NEB reaction buffer 3: 100mM Tris-HCl, 100mM $MgCl_2$, 1000mM NaCl, 10mM DTT, pH 7.0

10 x NEB reaction buffer 4: 200mM Tris-acetate, 100mM magnesium acetate, 500mM potassium acetate, 10mM DTT, pH 7.0

Reference Example 2: Phenol Extraction and Ethanol Precipitation

After the completion of an enzyme reaction with DNA, the reaction mixture was extracted with phenol for the purpose of inactivating the enzyme or recovering the DNA in the reaction mixture, wherein phenol preequilibrated with a buffer containing 10mM Tris-HCl(pH 8.0) and 1mM EDTA was used.

Phenol extraction was carried out by mixing equal volumes of the sample and the phenol with vigorous shaking; centrifuging the mixture at 15,000rpm for 5 minutes; and transferring the aqueous layer into a new tube. The above procedure was repeated three times.

The aqueous layer was, then, extracted with an equal volume of chloroform(chloroform:isobutanol = 24:1) and the aqueous layer was separated again; 0.1 volume of 3M sodium acetate and 2.5 volume of absolute ethanol were added thereto; and, the mixture was centrifuged at 15,000rpm and 4°C for 20 minutes after having left it at -70°C for 30 minutes or at -20°C for over 12 hours, to recover the nucleic acid.

Reference Example 3: Ligation Reaction

Ligation reaction of DNA was carried out by employing T4 DNA ligase and 10x ligation reaction buffer(0.5M Tris-HCl, pH 7.0, 0.1M $MgCl_2$, 0.2M DTT, 10mM ATP, 0.5mg/mℓ bovine serum albumin(BSA)) purchased from NEB. The reaction volume was generally 20μℓ, and 10 units of T4 ligase was used for the ligation of cohesive ends of DNA, while 100 units was used for the ligation of blunt ended DNAs.

The reaction was carried out at 16°C for 5 hours or at 4°C for over 14 hours; and, after the reaction was completed, the reaction mixture was heated at 65°C for 15 minutes to inactivate T4 DNA ligase.

Reference Example 4: Transformation of E. coli

Transformation of E. coli strains(e.g., E. coli HB101(ATCC 33694), E. coli W3110(ATCC 27325) or E. coli JM105(ATCC 47016)) was carried out by employing a method

known in the art, e.g., as described by Maniatis et al., in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y.(1982), or by Cohen in Proc. Natl. Acad. Sci. U.S.A. 69, 2110(1972).

Reference Example 5: Synthesis of Oligonucleotides

Oligonucleotides were synthesized by employing a DNA synthesizer(Applied Biosystems Inc., model No. 380B, U.S.A.) using automatic solid phase phosphoamidite chemistry.

The synthesized oligonucleotides were purified by using denaturing polyacrylamide gel(2M urea, 12% acrylamide and bis(29:1), 50mM Tris, 50mM boric acid, 1mM EDTA-Na_2) electrophoresis and C_{18} SEP-PAK(Waters Inc., U.S.A) column chromatography by using acetonitrile:water(50:50) as an eluent; and the amount was determined by measuring O.D. at 260nm.

Reference Example 6: Polymerase Chain Reaction(PCR)

To a mixture of 10 to 100ng of a template DNA, 10$\mu\ell$ of 10x Taq polymerase reaction buffer(10mM Tris-HCl, pH 8.3, 500mM KCl, 15mM $MgCl_2$, 0.1%(w/v) gelatin), 10$\mu\ell$ of a mixture of dNTP's(each of dGTP, dATP, TTP and dCTP is 10mM), 2μg of each primer(generally, 2 primers were used for a reaction, and in the case that 3 primers were used, the primer located in the middle was used in an amount of 0.02μg), and 0.5$\mu\ell$ of AmpliTaq DNA polymerase(Perkin Elmer Cetus, U.S.A.) was added distilled water in an amount to make a total volume of 100$\mu\ell$; and 50$\mu\ell$ of mineral oil was added thereto to protect the reaction mixture from evaporation.

The PCR was carried out by using a thermal cycler(Perkin Elmer Cetus, U.S.A.); and the thermal cycle was programmed to repeat 25 times or more, the cycle of: 95°C for 1 minute → 55°C for 1 minute → 72°C for 2 minutes, and, finally, the reaction was carried out at 72°C for 10 minutes.

After the reaction was completed, the mixture was extracted with phenol and the PCR products were recovered by

precipitation with ethanol; and, the precipitate was dissolved in 20μℓ of TE buffer solution(10mM Tris-HCl, 1mM EDTA, pH 7.5).

Reference Example 7: Preparation of HRP-conjugated anti-human IgG antibodies

Antibodies against the Fc region of human IgG (Immunovision Inc., Arizona, U.S.A., Cat. No. GHF-1001) were purified by chromatography using human IgG-attached sepharose CL-4B affinity column and protein-G column(Pharmacia LKB, Sweden) to obtain said antibodies with a purity over 90%. The obtained antibodies were labelled with horseradish peroxidase according to sodium periodate method described in Nakane, et al., J. Histochemcytochem. 22, 1084(1974) as follows:

To a solution of 5mg of horseradish peroxidase (Boehringer Mannheim, Germany, Cat. No. 814.393) dissolved in 1.2mℓ of distilled water(DW) was added 0.3mℓ of 0.1M sodium periodate solution in 10mM sodium phosphate buffer(pH 7.0); and the mixture was reacted at room temperature for 20 min. The resulting solution was dialyzed against 1mM sodium acetate buffer for 16 hours.

1.5mℓ of the resulting peroxidase solution was mixed with 1mℓ of 20mM sodium carbonate solution(pH 9.5) in which purified antibodies were dissolved to a concentration of 10mg/mℓ, and the mixture was reacted at room temperature for 2 hours. Then, 100μℓ of solution of sodium borohydride in DW (4mg/mℓ) was added thereto to remove the unreacted Schiff's base by a reduction reaction. The resulting solution was dialyzed against phosphate buffered saline overnight and then passed through sephadex G-200 column to remove free antibodies or peroxidases.

Example 1: Determination of epitope of non-structural 3 protein

<Step 1> Amplification of partial fragments of KHCV 897 DNA

(1-1) Preparation of primers

In order to amplify partial fragments of KHCV 897 DNA encoding non-structural 3 protein and to clone each into an expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized:

Primer P897T2: 5'-TGAGACTCCGCGGTGGTGCGGTGGAATTCATACCCGTT-3' comprising a recognition site of SacII and the 3916th to the 3936th nucleotides of KHCV-LBC1;

Primer P518T2: 5'-TGAGACTCCGCGGTGGTATCACCACAGGFCGCCCCTATC-3' comprising a recognition site of SacII and the 4195th to the 4216th nucleotides of KHCV-LBC1;

Primer P365T2: 5'-TGAGACTCCGCGGTGGTGCGGAGACGGCTGGAGCGCGG-3' comprising a recognition site of SacII and the 4348th to the 4369th nucleotides of KHCV-LBC1;

Primer P257T2: 5'-TGAGACTCCGCGGTGGTAACATTGGAGAGATTCCTTTC-3' comprising a recognition site of SacII and the 4447th to the 4468th nucleotides of KHCV-LBC1;

Primer P150T2: 5'-TGAGACTCCGCGGTGGTTTGTCCCTCGGAGTCAATGCT-3' comprising a recognition site of SacII and the 4564th to the 4585th nucleotides of KHCV-LBC1;

Primer P897SAL: 5'-GACTGGACTATTAACACGTATTACAGTCGATCAC-3' comprising a stop codon to terminate translation after the 4712nd nucleotide of KHCV-LBC1 and a recognition site of SalI;

Primer P652SAL: 5'-GACTGGACTATTACAGCTTTGCAGCGAGCTCGTC-3' comprising a stop codon to terminate translation after the 4562nd nucleotide of KHCV-LBC1 and a recognition site of SalI;

Primer P570SAL: 5'-GACTGGACTATTAGAGGGGGATGGCTTTGCCATA-3' comprising a stop codon to terminate translation after the

4487th nucleotide of KHCV-LBC1 and a recognition site of SalI;

Primer P430SAL: 5'-GACTGGACTATTATTGGTCCAGGACCGTGCCAAT-3' comprising a stop codon to terminate translation after the 4346th nucleotide of KHCV-LBC1 and a recognition site of SalI; and

Primer P290SAL: 5'-GACTGGACTATTAGGCGCCTGTGGTGATGGTCCT-3' comprising a stop codon to terminate translation after the 4208th nucleotide of KHCV-LBC1 and a recognition site of SalI.

(1-2) Polymerase chain reaction

8 different test tubes were prepared, which were provided with the primers as follows:

Tube A: Primer P897T2 2μg, Primer P652SAL 2μg
Tube B: Primer P897T2 2μg, Primer P570SAL 2μg
Tube C: Primer P897T2 2μg, Primer P430SAL 2μg
Tube D: Primer P897T2 2μg, Primer P290SAL 2μg
Tube E: Primer P150T2 2μg, Primer P897SAL 2μg
Tube F: Primer P257T2 2μg, Primer P897SAL 2μg
Tube G: Primer P365T2 2μg, Primer P897SAL 2μg
Tube H: Primer P518T2 2μg, Primer P897SAL 2μg

To each of the tubes were added 50ng of the plasmid ptrp-UB-KHCV897 comprising KHCV 897 DNA(ATCC 68640), 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

50μℓ of mineral oil was added to each of the reaction mixtures to prevent evaporation; and PCRs were carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(1-3) Separation and purification of PCR products

The PCR products obtained in the above (1-2) were subjected to 5% polyacrylamide gel electrophoresis. As a

result, it was confirmed that about 650bp of DNA in tube A, about 580bp of DNA in tube B, about 440bp of DNA in tube C, about 300bp of DNA in tube D, about 160bp of DNA in tube E, about 260bp of DNA in tube F, about 370bp of DNA in tube G, about 520bp of DNA in tube H were amplified, respectively. The DNAs were purified by the same polyacrylamide gel electrophoresis as above and named segment 652, segment 570, segment 430, segment 290, segment 150, segment 257, segment 365, and segment 518, respectively. The position of each fragment in KHCV 897 DNA and primers used for the preparation thereof are shown in Fig.4.

<Step 2> Preparation of expression vector

(2-1) Digestion of the segments and vector DNA with restriction endonucleases

2μg of each of DNA segments obtained in the <Step 1> was completely digested with SacII and SalI in NEB buffer solution 3 referred to in Reference Example 1. The resulting fragments were named fragments 652-T2/L, 570-T2/L, 430-T2/L, 290-T2/L, 150-T2/L, 257-T2/L, 365-T2/L, and 518-T2/L, respectively.

On the other hand, 2μg of plasmid ptrpH-UB-CORE14(ATCC 68642; see Korean Patent Laid-open Publication No. 93-683) was also completely digested with SacII and SalI in NEB buffer solution 3. The resulting mixture was subjected to 7% agarose gel electrophoresis to isolate 2.7kb fragment, which was named fragment ptrpH-UB-T2/L.

The above fragments were used in the ligation reactions as follows:

Ligation tube A was provided with 100ng of fragment 652-T2/L; ligation tube B was provided with 100ng of fragment 570-T2/L; ligation tube C was provided with 100ng of fragment 430-T2/L; ligation tube D was provided with 100ng of fragment 290-T2/L; ligation tube E was provided with 100ng of fragment 150-T2/L; ligation tube F was provided with 100ng of fragment 257-T2/L; ligation tube G was provided with 100ng of fragment 365-T2/L; and ligation tube H was provided with 100ng of fragment 518-

T2/L. To each of the tubes were added 50ng of fragment ptrpH-UB-T2/L, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

E. coli HB101(ATCC 33694) cell aliquots were transformed with each of the ligation mixture, respectively; and desired recombinant expression vectors were isolated therefrom, in accordance with Reference Example 4.

The vector containing fragment 652 was isolated and named ptrpH-UB-KHCV 652; the vector containing fragment 570 was isolated and named ptrpH-UB-KHCV 570; the vector containing fragment 430 was isolated and named ptrpH-UB-KHCV 430; the vector containing fragment 290 was isolated and named ptrpH-UB-KHCV 290; the vector containing fragment 150 was isolated and named ptrpH-UB-KHCV 150; the vector containing fragment 257 was isolated and named ptrpH-UB-KHCV 257; the vector containing fragment 365 was isolated and named ptrpH-UB-KHCV 365; and the vector containing fragment 518 was isolated and named ptrpH-UB-KHCV 518(see Fig. 5).

<Step 3> Expression of KHCV 897 DNA partial fragments

E. coli W3110(ATCC 37339) was transformed with each of the vectors prepared in the above <Step 2>.

Transformed E. coli cells were cultured with shaking in liquid Luria medium(6% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) containing 50μg/mℓ of ampicillin at 37°C for 12 hours. 3mℓ of the culture was transferred into 300 mℓ of M9 medium (40mM K_2HPO_4, 22mM KH_2PO_4, 8.5mM NaCl, 18.7mM NH_4Cl, 1% glucose, 0.1mM $MgSO_4$, 0.1mM $CaCl_2$, 0.4% casamino acid, 10μg/mℓ Vit. B_1, 40μg/mℓ ampicillin); and cultured with shaking for 4 hours at 37°C. When its O.D. value at 650nm reached 0.3, indole acrylic acid(IAA) was added to the culture to adjust the final concentration to be 50μg/mℓ. After 5 hours, the resulting culture was centrifuged at 11,000rpm for 25min. to collect the E. coli cell precipitates.

<Step 4> Identification of epitopes of KHCV 897 protein

Each of the cell precipitates obtained in the above <Step 3> was subjected to 15% SDS-PAGE by employing Laemmli's method (Nature 227, 680(1970)); and the gel was stained with Coomassie brilliant blue R250 to confirm the expression of the recombinant proteins. The result is shown in Fig. 6 A.

In Fig. 6 A, lane M represents the standard molecular size marker, lane 1 shows the products of E. coli having plasmid without any KHCV DNA fragment; lane 2 shows the products of E. coli transformed with ptrpH-UB-KHCV 897; lane 3 shows the products of E. coli transformed with ptrpH-UB-KHCV 290; lane 4 shows the products of E. coli transformed with ptrpH-UB-KHCV 430; lane 5 shows the products of E. coli transformed with ptrpH-UB-KHCV 570; lane 6 shows the products of E. coli transformed with ptrpH-UB-KHCV 652; lane 7 shows the products of E. coli transformed with ptrpH-UB-KHCV 518; lane 8 shows the products of E. coli transformed with ptrpH-UB-KHCV 365; lane 9 shows the products of E. coli transformed with ptrpH-UB-KHCV 257; and lane 10 shows the products of E. coli transformed with ptrpH-UB-KHCV 150.

The proteins separated on the gel were blotted onto a nitrocellulose filter(Bio-Rad Lab., pore size 0.22μm, CA, U.S.A.) by employing Towbin's method(Towbin, et al., Proc. Natl. Acad. Sci. U.S.A. 76, 4750(1979)). The filter was put in PBS(10mM phosphate, pH 7.0, 0.15M NaCl) containing 0.5% Tween 20; and shaken gently at room temperature for 2 hours to block the nonspecific binding of IgG to the proteins. The filter was put in IgG solution prepared by diluting IgG purified from Korean HCV patients with PBS containing 0.5% gelatin and 0.05% Tween 20 to adjust the final concentration to be 16μg/mℓ; and mildly shaken for 1 hour at room temperature to react the protein and IgG. The filter was then washed 4 times with PBS containing 0.2% Tween 20, each for 5 minutes. The filter was put in an anti-human IgG antibody solution prepared by diluting goat anti-human IgG labeled with horseradish peroxidase (goat anti-human IgG-HRP, Bio-Rad Lab., CA., U.S.A.) with 500-fold volume of PBS

containing 0.5% gelatin and 0.05% Tween 20; and shaken at room temperature for 1 hour. The filter was washed 4 times with PBS containing 0.2% Tween 20, each for 5 minutes; and then, twice with 50mM Tris buffer solution(pH 7.0). To the filter were added 50mM Tris buffer solution containing 400μg/mℓ 4-chloro-1-naphthol and 0.03% hydrogen peroxide to develop a color reaction. The results from the above western blotting are shown in Fig. 6 B. In Fig. 6 B, lane M and lanes 1 to 10 represent the same samples as Fig. 6 A; and lanes 5 to 10 show positive results, while lanes 3 and 4 show negative results.

Therefore, as can be seen from Figs. 6 A and 6 B, the epitope of KHCV 897 protein exists in carboxyl terminal region of KHCV 897 protein(KHCV 365 protein), i.e., it is encoded by 366 base pairs consisting of the 4348th to the 4713rd nucleotides of KHCV-LBC1. However, said KHCV 365 protein exhibits lower immunoreactivity than KHCV 897 protein. From this fact, it can be presumed that amino acids in the N-terminal of KHCV 365 protein cannot serve as epitope when they are expressed as N-terminal amino acids. Therefore, KHCV 518 protein which comprise said KHCV 365 protein and extencted N-terminal amino acids and has immunoreactivity similar to that of KHCV 897 protein were used hereinafter for preparing HCV diagnostic agents.

Example 2: Determination of epitopes of HCV envelope protein

<Step 1> Amplification of the partial fragments of HCV envelope gene

(1-1) Preparation of primers

In order to amplify KHCV envelope gene fragments and to clone each into an expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized:

Primer PE1T2: 5'-TGAGACTCCGCGGTGGTTATGAAGTGGGCAACGCGTCC-3'
comprising a recognition site of SacII and the 916th to the

937th nucleotides of KHCV-LBC1;

Primer PE1DT2: 5'-TGAGACTCCGCGGTGGTGACTTGCTCGTTGGGGTAGCT-3' comprising a recognition site of SacII and the 1129th to the 1149th nucleotides of KHCV-LBC1;

Primer PE1EGT2: 5'-TGAGACTCCGCGGTGGTGTTTCCCAGCTGTTCACCTTC-3' comprising a recognition site of SacII and the 1201st to the 1221st nucleotides of KHCV-LBC1;

Primer PE1FT2: 5'-TGAGACTCCGCGGTGGTACAACAGCCCTAGTGGTATCG-3' comprising a recognition site of SacII and the 1327th to the 1347th nucleotides of KHCV-LBC1;

Primer PE1AXHO: 5'-AAAAAACTCGAGTTAGACATGGCGTCGCAATGTCGT-3' comprising a stop codon to terminate translation after the 1128th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE1BXHO: 5'-AAAAAACTCGAGTTAAAGGAAAACAGATCCGCAGAG-3' comprising a stop codon to terminate translation after the 1200th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE1CDEXHO: 5'-AAAAAACTCGAGTTAAGGCGACCAGTTCATCATCAT-3' comprising a stop codon to terminate translation after the 1326th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE1XHO: 5'-AAAAAACTCGAGTTACCCTGTCACGTGGGTGGTTCC-3' comprising a stop codon to terminate translation after the 2835th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE2T2: 5'-TGAGACTCCGCGGTGGTGGGGCGCAAGGTCGGGCCGCT-3' comprising a recognition site of SacII and the 1509th to the 1529th nucleotides of KHCV-LBC1;

Primer PE2BT2: 5'-TGAGACTCCGCGGTGGTGGTCCCATCACTTACACTGAG-3' comprising a recognition site of SacII and the 1749th to the 1769th nucleotides of KHCV-LBC1;

Primer PE2DFT2: 5'-TGAGACTCCGCGGTGGTGGCACTGGGTTCACCAAGACA-3' comprising a recognition site of SacII and the 2010th to the 2030th nucleotides of KHCV-LBC1;

Primer PE2ET2: 5'-TGAGACTCCGCGGTGGTACTCGGGGAGAGCGTTGTGAC-3' comprising a recognition site of SacII and the 2280th to the 2300th nucleotides of KHCV-LBC1;

Primer PE2AXHO: 5'-AAAAAACTCGAGTTACCACCCCTGCGCGAATGTATC-3' comprising a stop codon to terminate translation after the 1748th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE2BCXHO: 5'-AAAAAACTCGAGTTAATTCATCCAGGTACAACCGAA-3' comprising a stop codon to terminate translation after the 2009th nucleotide of KHCV-LBC1, and a recognition site of XhoI;

Primer PE2DXHO: 5'-AAAAAACTCGAGTTACCAGTTGCATGCGGCGTCGAG-3' comprising a stop codon to terminate translation after the 2279th nucleotide of KHCV-LBC1, and a recognition site of XhoI; and

Primer PE2XHO: 5'-AAAAAACTCGAGTTACGCGTCCGCCAGAAGAAGGAAGAG-3' comprising a stop codon to terminate translation after the 2528th nucleotide of KHCV-LBC1, and a recognition site of XhoI.

(1-2) Polymerase chain reaction

14 different test tubes were prepared, which were provided with the primers as follows:

Tube A: Primer PE1T2 2μg, Primer PE1XHO 2μg

Tube B: Primer PE1T2 2μg, Primer PE1AXHO 2μg

Tube C: Primer PE1T2 2μg, Primer PE1BXHO 2μg

Tube D: Primer PE1T2 2μg, Primer PE1CDEXHO 2μg
Tube E: Primer PE1DT2 2μg, Primer PE1CDEXHO 2μg
Tube F: Primer PE1EGT2 2μg, Primer PE1CDEXHO 2μg
Tube G: Primer PE1FT2 2μg, Primer PE1XHO 2μg
Tube H: Primer PE1EGT2 2μg, Primer PE1XHO 2μg
Tube I: Primer PE2T2 2μg, Primer PE2AXHO 2μg
Tube J: Primer PE2BT2 2μg, Primer PE2BCXHO 2μg
Tube K: Primer PE2T2 2μg, Primer PE2BCXHO 2μg
Tube L: Primer PE2DFT2 2μg, Primer PE2DXHO 2μg
Tube M: Primer PE2ET2 2μg, Primer PE2XHO 2μg
Tube N: Primer PE2DFT2 2μg, Primer PE2XHO 2μg

To each of the tubes were added each 50ng of the vector ptrpH-UB-E1 comprising envelope 1 gene(ATCC 68878) for tubes A to H and the vector pYLBC-A/G-UB-E2N and pYLBC-A/G-UB-E2C comprising envelope 2 gene(ATCC 69886 and 74117) for tubes I to N as a template, 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2m dGTP, 2m dATP, 2mM TTP, 2mM dCTP), 2.5μℓ of 10 unit Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To each of the reaction mixtures was added 50μℓ of mineral oil to prevent evaporation; and PCRs were carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(1-3) Separation and purification of PCR products

The PCR products obtained in the above (1-2) were subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 600bp of DNA in tube A, about 220bp of DNA in tube B, about 300bp of DNA in tube C, about 410bp of DNA in tube D, about 200bp of DNA in tube E, about 110bp of DNA in tube F, about 190bp of DNA in tube G, about 300bp of DNA in tube H, about 210bp of DNA in tube I, about 300bp of DNA in tube J, about 505bp of DNA in tube K, about 290bp of DNA in tube L, about 240bp of DNA in tube M, and about 520bp of DNA in tube N were amplified, respectively. The DNAs were purified by the same polyacrylamide gel electrophoresis as above and named segment

E1, segment E1A, segment E1B, segment E1C, segment E1D, segment E1E, segment E1F, segment E1G, segment E2A, segment E2B, segment E2C, segment E2D, segment E2E, and segment E2F. The positions of each segments of the envelope gene and primers used for the preparation thereof are shown in Fig. 7.

<Step 2> Preparation of expression vector

2μg of each of DNA segments obtained in the (1-3) of <Step 1> was completely digested with SacII and XhoI in NEB buffer solution 3 referred to in Reference Example 1.

Each of 14 ligation tubes was provided with 100ng of DNA segments obtained above. To each of the tubes were added 50ng of fragment ptrpH-UB-T2/L which was obtained in the <Step 2> of Example 1, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

Fourteen E. coli W3110(ATCC 37339) cell aliquots were transformed with each of the ligation mixtures, respectively.

The vector containing segment E1 was isolated and named ptrpH-UB-E1; the vector containing segment E1A was isolated and named ptrpH-UB-E1A; the vector containing segment E1B was isolated and named ptrpH-UB-E1B; the vector containing segment E1C was isolated and named ptrpH-UB-E1C; the vector containing segment E1D was isolated and named ptrpH-UB-E1D; the vector containing segment E1E was isolated and named ptrpH-UB-E1E; the vector containing segment E1F was isolated and named ptrpH-UB-E1F; the vector containing segment E1G was isolated and named ptrpH-UB-E1G; the vector containing segment E2A was isolated and named ptrpH-UB-E2A; the vector containing segment E2B was isolated and named ptrpH-UB-E2B; the vector containing segment E2C was isolated and named ptrpH-UB-E2C; the vector containing segment E2D was isolated and named ptrpH-UB-E2D; the vector containing segment E2E was isolated and named ptrpH-UB-E2E; and the vector containing segment E2F was isolated and named ptrpH-UB-E2F(see Fig. 8).

<Step 3> Expression of envelope gene segments

E. coli W3110(ATCC 37339) cells transformed with each of the plasmids containing envelope gene fragments prepared in the above <Step 2> were cultured in the same manner as in <Step 3> of Example 1; and then centrifuged to collect the E. coli cell precipitates.

<Step 4> Identification of epitopes of envelope protein

Epitopes of envelope protein were identified by employing the cell precipitates of <Step 3> in the same manner as in <Step 4> of Example 1; and the result is shown in Fig. 9, wherein A is the result of SDS-PAGE and B is the result of western blotting.

In Fig. 9, lane 1 shows the products of E. coli having plasmid without any envelope gene segment; lane 2 shows the products of E. coli transformed with ptrpH-UB-E1; lane 3 shows the products of E. coli transformed with ptrpH-UB-E1A; lane 4 shows the products of E. coli transformed with ptrpH-UB-E1B; lane 5 shows the products of E. coli transformed with ptrpH-UB-E1C; lane 6 shows the products of E. coli transformed with ptrpH-UB-E1D; lane 7 shows the products of E. coli transformed with ptrpH-UB-E1E; lane 8 shows the products of E. coli transformed with ptrpH-UB-E1F; lane 9 shows the products of E. coli transformed with ptrpH-UB-E1G; lane 10 shows the products of E. coli transformed with ptrpH-UB-E2A; lane 11 shows the products of E. coli transformed with ptrpH-UB-E2B; lane 12 shows the products of E. coli transformed with ptrpH-UB-E2C; lane 13 shows the products of E. coli transformed with ptrpH-UB-E2D; lane 14 shows the products of E. coli transformed with ptrpH-UB-E2E; and lane 15 shows the products of E. coli transformed with ptrpH-UB-E2F.

The result of western blotting analysis employing the E. coli cells transformed with a plasmid comprising various envelope gene segments to confirm the specificity thereof against the anti-KHCV antibodies obtained from the serum of

Korean hepatitis C patient is shown in Table I below, as well as in Fig. 9B.

Table I. Result of Western Blotting analysis employing KHCV envelope protein fragments

Lane	protein fragment	position (No. of a.a.)	Western blotting signal
2	UBE1	E1 1-198	+
3	UBE1A	E1 1-72	-
4	UBE1B	E1 1-99	-
5	UBE1C	E1 1-136	(+)
6	UBE1D	E1 72-136	(+)
7	UBE1E	E1 100-136	((+))
8	UBE1F	E1 136-198	+
9	UBE1G	E1 100-198	+
10	UBE2A	E2 1-67	(+)
11	UBE2B	E2 68-167	-
12	UBE2C	E2 1-167	(+)
13	UBE2D	E2 168-262	-
14	UBE2E	E2 263-340	+
15	UBE2F	E2 168-340	+

* Western signal + : positive
(+) weak ((+)) very weak
- : negative

As can be seen from Table I and Fig. 9 B, the lanes 9, 10 and 14 which represent the products of E. coli transformed with a plasmid comprising envelope gene segments E1G, E2A and E2E, respectively, show positive signals, while the other lanes which represent the products of E. coli transformed with a plasmid comprising envelope gene segments E1A, E1B, E2B, E2D, etc. show negative signals.

Therefore, it has been found that epitopes of envelope protein exist in the carboxyl terminal region of KHCV envelope 1 protein which was expressed from the 309 base pairs corresponding to 1201st to 1509th nucleotides of KHCV-LBC1(E1G protein); in the amino terminal region of KHCV envelope 2 protein which was expressed from the 240 base pairs corresponding to 1510th to 1749th nucleotides of KHCV-LBC1(E2A protein); and in the carboxyl terminal region of KHCV envelope 2 protein which was expressed from the 249 base pairs corresponding to 2281st to 2529th nucleotides of KHCV-

LBC1(E2E protein)(see Fig. 2 for the amino acid and nucleotide sequences of E1G, E2A and E2E protein).

The following Examples 3 to 5 show the preparation of recombinant proteins comprising more than one epitope of HCV.

Example 3: Preparation of KHCV UB CORE518 protein

<Step 1> Amplification of KHCV 518 DNA

(1-1) Preparation of primers

In order to amplify KHCV 518 DNA(which consists of the region from the 4196th to the 4713rd nucleotides of KHCV-LBC1) and to clone it into an expression vector comprising ubiquitin gene and KHCV CORE14 DNA(ATCC 68642; see Korean Patent Publication No. 93-683) under the control of trp promotor, the following primers were synthesized.

Primer PK518T2:
5'-TGAGACTCCGCGGTGGTGGAGGAGGAGGAGGAGGAATCACCACAG
GCGCCCCTATC-3'
comprising a recognition site of SacII, 6 glycine codons, and the 4195th to the 4215th nucleotides of KHCV-LBC1; and

Primer PK518SAL: 5'-AAAAAAGTCGACTATTAACACGTATTACAGTCGATCAC-3'
comprising a stop codon to terminate translation after the 4713rd nucleotide of KHCV-LBC1, and a recognition site of SalI.

(1-2) Polymerase chain reaction

A test tube was provided with the primer PK518T2 2μg and primer PK518SAL 2μg. To the tube were added 50ng of KHCV-LBC1 DNA(ATCC 75008), 10$\mu\ell$ of 10x polymerase buffer solution, 10$\mu\ell$ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100$\mu\ell$.

To the reaction mixture was added 50$\mu\ell$ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(1-3) Separation and purification of PCR product

The PCR product obtained in the above (1-2) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 520bp of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as (1-2) above and named fragment GLYK518.

<Step 2> Preparation of expression vector

2μg of plasmid ptrpH-UB-CORE14(ATCC 68642; see Korean Patent Laid-open Publication No. 93-683) was completely digested with SalI in NEB buffer solution 3 referred to in Reference Example 1, and then partially digested with SacII under the same condition. The resulting mixture was subjected to 7% agarose gel electrophoresis to isolate 3.0kb fragment, which was named fragment ptrpH-UB-CORE(T2)/L.

2μg of the fragment GLYK518 obtained in the (1-3) of <Step 1> was completely digested with SacII and SalI in NEB buffer solution 3 referred to in Reference Example 1.

A reaction tube was provided with 100ng of DNA fragment obtained above. To the tube were added 50ng of the fragment ptrpH-UB-CORE(T2)/L, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

E. coli W3110(ATCC 37339) was transformed with the ligation mixture to obtain recombinant E. coli transformant containing plasmid ptrpH-UB-CORE518 comprising the fragment GLYK518(see Fig. 10) connected with ubiquitin gene and KHCV CORE 14 DNA in an open reading frame("CORE 518 DNA").

<Step 3> Expression of CORE518 DNA

E. coli W3110 cells transformed with the plasmid ptrpH-UB-CORE518 prepared in the above <Step 2> were cultured in the same manner as in <Step 3> of Example 1; and then centrifuged to collect the E. coli cell precipitates.

The reactivity of the expressed CORE518 protein with a serum taken from a hepatitis C patient was confirmed by employing the above cell precipitates in the same manner as in <Step 4> of Example 1; and the result is shown in Fig. 11, wherein A is the result of SDS-PAGE and B is the result of Western blotting.

In Fig. 11, lanes 1 and 4 show the products of E. coli not having plasmid ptrpH-UB-CORE518; lanes 2 and 5 show the products of E. coli transformed with ptrpH-UB-CORE518; and lane 3 shows the standard molecular size markers, i.e., 70, 43, 29, 18 and 14 kilodaltons from the top of the gel.

<Step 4> Purification of UBCORE518 protein

(4-1) Cell disruption and removal of soluble proteins

3g of E. coli cell precipitate obtained in <Step 3> was suspended in 40mℓ of buffer 1(20mM Tris, pH 8.0, 1mM EDTA, 10mM ß-mercaptoethanol, 1mM phenyl methyl sulfonyl fluoride, 1μg/mℓ pepstatin A). Lysozyme was added to the suspension to adjust the final concentration to be 0.2mg/mℓ, and the resulting solution was incubated on ice for 30 minutes. The resultant was subjected to ultrasonication in an ice bath for 15 minutes with an ultrasonicater(HEAT SYSTEMS ULTRASONICS INC., W225, U.S.A.) at an output of 80% and 50% duty-cycle to disrupt the cell and obtain a homogenate of E. coli cells.

The cell homogenate obtained in the above was centrifuged at 15,000 rpm for 25 minutes with a centrifuge(Beckman J2-21, Rotor JA 20) to remove dissolved proteins and obtain insoluble precipitate.

(4-2) Washing of insoluble precipitate

The precipitate obtained in (4-1) was suspended in 4mℓ of buffer 2(20mM Tris, pH 8.0, 1mM EDTA, 10mM ß-mercaptoethanol) containing 1% Triton X-100. The suspension was stirred at room temperature for 30 minutes and centrifuged at 15,000 rpm for 25 minutes with a centrifuge(Beckman J2-21, Rotor JA 20) to remove dissolved proteins and obtain insoluble precipitate.

(4-3) Dissolution of insoluble precipitate

The insoluble precipitate of (4-2) was suspended in 100mℓ of buffer 3(50mM Tris, pH 9.0, 1mM EDTA, 10mM ß-mercapto-ethanol) containing 4M urea. The suspension was stirred at room temperature for 2 hours and centrifuged to remove insoluble precipitate and obtain the supernatant.

(4-4) DEAE-Sepharose ion exchange chromatography

The supernatant obtained in (4-3) was passed through DEAE-Sepharose column(Pharmacia, 1.25cm x 4cm) equilibrated with the above buffer 3 at a flow rate of 4mℓ/min.; and same buffer was added to elute free proteins remains in column. Then, 200mℓ of buffer 3 having a concentration gradient of 0 to 0.3M NaCl was added at a flow rate of 4mℓ/min. to elute the bound proteins and collect the eluate by 2mℓ fractions. The protein fractions were subjected to 15% SDS-PAGE to collect the fractions comprising UBCORE518 protein.

(4-5) FPLC-MONO S chromatography

The protein fractions comprising UBCORE518 protein collected in (4-4) were concentrated to a volume of 20mℓ with YM10 ultrafiltration membrane (Amicon, U.S.A.). The concentrate was passed over G-25 column(Pharmacia, 2.5cm x 90cm) equilibrated with the buffer 4(50mM phosphate, pH 6.0, 1mM EDTA, 10mM ß-mercaptoethanol) containing 4M urea. The eluate was in turn passed over FPLC-Mono S column(Pharmacia, HR 5/5, 0.5cm x 5cm) equilibrated with the same buffer at a flow rate of 0.7mℓ/min.; and same buffer was added to elute free proteins remained in column. Then, same buffer containing 0.2M of NaCl was added to elute the bound proteins. 200mℓ of buffer 5(10mM phosphate, pH 7.0) having a linear concentration gradient of 0.2 to 0.4M NaCl was added to elute the bound proteins and collect the eluate by 0.7mℓ fractions. The protein fractions were subjected to 15% SDS-PAGE to collect the fractions comprising UBCORE518 protein having a purity of at least 95%; and the antigenic specificity of the purified protein was confirmed by employing western blotting analysis.

Example 4: Preparation of KHCV UB NS4E1E2 protein

<Step 1> Expression vector for KHCV NS4E DNA

(1-1) Preparation of primers

In order to prepare KHCV NS4E DNA(which consists of the region from the 5422nd to the 5547th nucleotides of KHCV-LBC1) and to clone it into an expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized:

Primer PNS4ET2: 5'-TGAGACTCCGCGGTGGTATCATCCCCGATAGGGAAGTT-3' comprising a recognition site of SacII and the 5422nd to the 5442nd nucleotides of KHCV-LBC1; and

Primer PNS4ESAL: 5'-AAAAAAGTCGACTATTACAACCCGAGCGCCTTCTGTTT-3' comprising a stop codon to terminate translation after the 5547th nucleotide of KHCV-LBC1, and a recognition site of SalI.

(1-2) Polymerase chain reaction

A test tube was provided with the primer PNS4ET2 2μg and primer PNS4ESAL 2μg. To the tube were added 50ng of KHCV-LBC1 DNA(ATCC 75008), 10$\mu\ell$ of 10x polymerase buffer solution, 10$\mu\ell$ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100$\mu\ell$.

To the reaction mixture was added 50$\mu\ell$ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(1-3) Separation and purification of PCR product

The PCR product obtained in the above (1-2) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 130bp of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment NS4E.

<Step 2> Preparation of expression vector

2μg of fragment NS4E obtained in the above (1-3) was completely digested with SacII and SalI in NEB buffer solution 3 referred to in Reference Example 1.

A ligation tube was provided with 100ng of DNA fragment obtained above. To the tube were added 50ng of fragment ptrpH-UB-T2/L obtained in <Step 2> of Example 1, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

E. coli W3110(ATCC 37339) was transformed with the ligation mixture to obtain recombinant E. coli cell containing plasmid ptrpH-UB-NS4E comprising fragment NS4E(Fig. 12).

<Step 3> Preparation of KHCV E1E2 protein

(3-1) Preparation of primers

In order to amplify KHCV E1E2 gene which comprising the epitopes of HCV envelope protein and to clone it into an expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized:

Primer PE2ET2: 5'-TGAGACTCCGCGGTGGTACTCGGGGAGAGCGTTGTGAC-3' comprising a recognition site of SacII and the 2281st to the 2298th nucleotides of KHCV-LBC1;

Primer PE2EGE1G:
5'-TTCCTTCTTCTGGCGGACGCGGTTTCCCAGCTGTTCACCTTC-3'
comprising the region from the 2509th to the 2529th nucleotides of KHCV-LBC1, which is the 3'-end region of E2E DNA, and the region from the 1201st to the 1221st nucleotides of KHCV-LBC1 which is the 5'-end region of E1G gene; and

Primer PE2AXHO: 5'-AAAAAACTCGAGTTACCACCCCTGCGCGAATGTATC-3' comprising a stop codon to terminate translation after the 1749th nucleotide of KHCV-LBC1, and a recognition site of

XhoI.

(3-2) Polymerase chain reaction

A test tube was provided with the primer PE2EGE1G 2μg and primer PE2AXHO 2μg. To the tube were added 50ng of KHCV-LBC1 DNA(ATCC 75008) as a template, 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To the reaction mixture was added 50μℓ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(3-3) Separation and purification of PCR product

The PCR product obtained in the above (3-2) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 550bp of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment GE1GE2A.

(3-4) Second Polymerase chain reaction

A test tube was provided with the primer PE2ET2 2μg and primer PE2AXHO 2μg. To the tube were added 50ng of plasmid pYLBC-A/G-UBE2C(ATCC 74117, see Korean Patent Laid-open Publication No. 93-683) and fragment GE1GE2A obtained in the above (3-3) as templates, 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To the reaction mixture was added 50μℓ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(3-5) Separation and purification of PCR product

The PCR product obtained in the above (3-4) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 800bp of DNA was

amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment E1E2.

(3-6) Preparation of expression vector

2μg of DNA fragment obtained in (3-5) was completely digested with SacII and XhoI in NEB buffer solution 3 in accordance with Reference Example 1.

A tube was provided with 100ng of DNA fragment obtained above. To the tube were added 50ng of fragment ptrpH-UB-T2/L obtained in <Step 2> of Example 1, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

E. coli W3110(ATCC 37339) was transformed with the ligation mixture to obtain recombinant E. coli cell containing plasmid ptrpH-UB-E1E2 comprising the fragment E1E2(Fig. 13).

(3-7) Expression of E1E2 DNA

E. coli W3110(ATCC 37339) cells transformed with the plasmid ptrpH-UB-E1E2 prepared in the above (3-6) were cultured in the same manner as in <Step 3> of Example 1; and then centrifuged to collect the E. coli cell precipitates.

(3-8) Confirmation of expressed UBE1E2 protein and reactivity thereof with a serum taken from a hepatitis C patient

Expression of UBE1E2 protein and their reactivity with a serum taken from a hepatitis C patient were confirmed by employing the cell precipitates of (3-7) in the same manner as in <Step 4> of Example 1; and the result is shown in Fig. 14, wherein A is the result of SDS-PAGE and B is the result of Western blotting.

In Figs. 14 A and B, lanes 1 and 4 show the products of E. coli not having plasmid ptrpH-UB-E1E2; lanes 2 and 5 show the products of E. coli transformed with ptrpH-UB-E1E2; and lane 3 shows the standard molecular size markers, i.e., 43, 29, 18 and 14 kilodaltons from the top of the gel.

<Step 4> Preparation of KHCV NS4E1E2 protein

<Step 4-A> Amplification of NS4E1E2 gene

(4-A-1) Preparation of primers

In order to amplify a gene which encodes the epitopes of HCV envelope protein and a part of KHCV NS4E DNA and to clone it into an expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized.

Primer PNS4ET2: 5'-TGAGACTCCGCGGTGGTATCATCCCCGATAGGGAAGTT-3' comprising a recognition site of SacII and the 5422nd to the 5442nd nucleotides of KHCV-LBC1;

Primer PNS4EGE2C3: 5'-CAGAAGGCGCTCGGGTTGCCAGGAGGAGGAGGTGGTA CTCGGGGAGAGCGTTGT-3'
comprising the region from the 5530th to the 5547th nucleotides of KHCV-LBC1, which is the 3'-end region of NS4E DNA, one proline codon, six glycine codons and the region from the 2281st to the 2298th nucleotides of KHCV-LBC1, which is the 5'-end region of E2E gene, in that order; and

Primer PE2AXHO: 5'-AAAAAACTCGAGTTACCACCCCTGCGCGAATGTATC-3' comprising a stop codon to terminate translation after the 1749th nucleotide of KHCV-LBC1, and a recognition site of XhoI.

(4-A-2) Polymerase chain reaction

A test tube was provided with 2μg of primer PNS4EGE2C3 and 2μg of primer PE2AXHO. To the tube were added 50ng of ptrpH-UB-E1E2((3-6) of <Step 3>) as a template, 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To the reaction mixture was added 50μℓ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25

times the same thermal cycles as in Reference Example 6.

(4-A-3) Separation and purification of PCR product

The PCR product obtained in the above (4-A-2) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 800bp of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment GENVEPI-III.

(4-A-4) Second Polymerase chain reaction

A test tube was provided with 2μg of primer PNS4ET2 and 2μg of primer PE2AXHO. To the tube were added 50ng of plasmid ptrpH-UB-NS4E obtained in the above <Step 1> as a template, 50ng of fragment GENVEPI-III obtained in the above (4-A-2), 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To the reaction mixture was added 50μℓ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(4-A-5) Separation and purification of PCR product

The PCR product obtained in the above (4-A-4) was subjected to 5% polyacrylamide gel electrophoresis. As a result, it was confirmed that about 920bp of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment NS4E1E2.

<Step 4-B> Preparation of expression vector

2μg of the DNA fragment obtained in (4-A-5) of <Step 4-A> was completely digested with SacII and XhoI in NEB buffer solution 3 referred to in Reference Example 1.

A tube was provided with 100ng of DNA fragment obtained above. To the tube were added 50ng of the fragment ptrpH-UB-T2/L obtained in <Step 2> of Example 1, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The

ligation was carried out at 16°C for 12 hours.

E. coli W3110(ATCC 37339) was transformed with the ligation mixture to obtain recombinant E. coli cell containing plasmid ptrpH-UB-NS4E1E2 comprising the fragment NS4E1E2(Fig. 15).

<Step 4-C> Expression of the fragment NS4E1E2 DNA

E. coli W3110(ATCC 37339) transformant harboring plasmid ptrpH-UB-E1E2 prepared in the above <Step 4-B> were cultured in the same manner as in <Step 3> of Example 1; and then centrifuged to collect the E. coli cell precipitates.

<Step 4-D> Confirmation of expressed UBNS4E1E2 protein and reactivity thereof with a serum taken from a hepatitis C patient

Production of UBNS4E1E2 protein in E. coli and their reactivity with a serum taken from a hepatitis C patient were confirmed by employing the cell precipitates of <Step 4-C> in the same manner as in <Step 4> of Example 1; and the result is shown in Fig. 16, wherein A is the result of SDS-PAGE and B is the result of western blotting.

In Figs. 16 A and B, lanes 1 and 4 show the products of E. coli not having plasmid ptrpH-UB-NS4E1E2; lanes 2 and 5 show the products of E. coli transformed with ptrpH-UB-NS4E1E2; and lane 3 shows the standard molecular size markers, i.e., 92, 70, 43, 29 and 18 kilodaltons from the top of the gel.

<Step 4-E> Purification of UBNS4E1E2 protein

(4-E-1) Cell disruption and removal of soluble proteins

2g of E. coli cell precipitate obtained in <Step 4-C> was treated as in <Step 4>(4-1) of Example 3 to disrupt the cell and obtain insoluble precipitate therefrom.

(4-E-2) Washing of insoluble precipitate

The precipitate obtained in (4-E-1) was treated as in <Step 4>(4-2) of Example 3 to remove dissolved proteins and

obtain insoluble precipitate.

(4-E-3) Washing with 4M urea

The insoluble precipitate of (4-E-2) was suspended in 30mℓ of buffer 2 containing 4M urea. The suspension was stirred at room temperature for 2 hours and centrifuged at 15,000rpm with a centrifuge(Beckman J2-21, Rotor JA 20) to remove dissolved proteins and obtain insoluble precipitate.

(4-E-4) Washing with 6M guanidine chloride

The insoluble precipitate of (4-E-3) was suspended in 30mℓ of buffer 2 containing 6M guanidine chloride. The suspension was stirred at room temperature for 2 hours and centrifuged at 15,000rpm with a centrifuge(Beckman J2-21, Rotor JA 20) to remove dissolved proteins and obtain insoluble precipitate.

(4-E-5) Dissolution of precipitate with 1% SDS

The insoluble precipitate of (4-E-4) was suspended in 10mℓ of PBS(10mM phosphate, pH 7.0, 150mM NaCl) containing 1% SDS. The suspension was stirred at room temperature for 12 hours and centrifuged at 15,000rpm with a centrifuge(Beckman J2-21, Rotor JA 20) to remove insoluble precipitate and obtain supernatant.

(4-E-6) S-300 gel filtration chromatography

10mℓ of the supernatant obtained in (4-E-5) were concentrated to a volume of 4mℓ with YM10 ultrafiltration membrane(Amicon, U.S.A.) and then, centrifuged at 15,000rpm for 25 minutes with a centrifuge(Beckman J2-21, Rotor JA 20) to remove insoluble precipitate and obtain supernatant. The supernatant was subjected to gel filtration chromatography with S-300 resin column(Pharmacia LKB, 2.5cm x 90cm) equilibrated with PBS containing 0.1% SDS at a flow rate of 40mℓ/hour. The eluted protein was collected by 2mℓ fractions and subjected to 15% SDS-PAGE to collect the fractions comprising UBNS4E1E2 protein having a purity of at least 90%; and the antigenic specificity of the purified protein was

confirmed by employing a western blotting analysis.

Example 5: Preparation of KHCV NS5-1.2 protein

<Step 1> Amplification of NS5-1.2 DNA

(1-1) Preparation of primers

In order to amplify KHCV NS5-1.2 DNA(which consists of the region from the 6649th to the 7824th nucleotides of KHCV-LBC1) and to clone it into an E. coli expression vector comprising ubiquitin gene under the control of trp promotor, the following primers were synthesized.

Primer PNS5T2: 5'-TGAGACTCCGCGGTGGTACGGGCATGACCACTGACAAC-3' comprising a recognition site of SacII and the 6649th to the 6669th nucleotides of KHCV-LBC1; and

Primer PNS5-1.2SAL:
5'-AAAAAAGTCGACTATTACGCCTTCCCCTTCATCTCCTT-3'
comprising a stop codon to terminate translation after the 7824th nucleotide of KHCV-LBC1, and a recognition site of SalI.

(1-2) Polymerase chain reaction

A test tube was provided with 2μg of primer PNS5T2 and 2μg of primer PNS5-1.2SAL. To the tube were added 50ng of KHCV-LBC1 DNA(ATCC 75008) as a template, 10μℓ of 10x polymerase buffer solution, 10μℓ of 2mM dNTP(2mM dGTP, 2mM dATP, 2mM TTP, 2mM dCTP), 2.5 unit of Taq polymerase; and distilled water was added thereto to adjust the total volume to be 100μℓ.

To the reaction mixture was added 50μℓ of mineral oil to prevent evaporation; and PCR was carried out by repeating 25 times the same thermal cycles as in Reference Example 6.

(1-3) Separation and purification of PCR product

The PCR product obtained in the above (1-2) was subjected to 5% polyacrylamide gel electrophoresis. As a

result, it was confirmed that about 1.2Kb of DNA was amplified. The DNA was purified by the same polyacrylamide gel electrophoresis as above and named fragment NS5-1.2.

<Step 2> Preparation of expression vector

2μg of DNA fragment obtained in (1-3) of <Step 1> was completely digested with SacII and SalI in NEB buffer solution 3 referred to in Reference Example 1.

A tube was provided with 100ng of DNA fragment obtained above. To the tube were added 50ng of fragment ptrpH-UB-T2/L obtained in <Step 2> of Example 1, 2μℓ of 10x ligation buffer solution, 10 units of T4 DNA ligase; and distilled water was added to adjust the total volume to be 20μℓ. The ligation was carried out at 16°C for 12 hours.

E. coli W3110(ATCC 37339) was transformed with the ligation mixture to obtain recombinant E. coli cell containing plasmid ptrpH-UB-NS5-1.2 comprising fragment NS5-1.2(Fig. 17).

<Step 3> Expression of the fragment NS5-1.2 DNA

E. coli W3110(ATCC 37339) cells transformed with the plasmid ptrpH-UB-NS5-1.2 prepared in the above <Step 2> were cultured in the same manner as in <Step 3> of Example 1; and then centrifuged to collect the E. coli cell precipitates.

<Step 4> Confirmation of expressed KHCV UBNS5-1.2 protein and reactivity thereof with a serum taken from a hepatitis C patient

Production of UBNS5-1.2 protein in E. coli and their reactivity with a serum taken from a hepatitis C patient were confirmed by employing the cell precipitates of <Step 3> in the same manner as in <Step 4> of Example 1; and the result is shown in Fig. 18, wherein A is the result of SDS-PAGE and B is the result of western blotting.

In Figs. 18 A and B, lane 1 shows the products of E.

coli not having plasmid ptrpH-UB-NS5-1.2; and lanes 2 and 3 show the products of E. coli transformed with ptrpH-UB-NS5-1.2.

<Step 5> Comparison of reactivity of KHCV UBNS5-1.2 protein with a serum taken from a hepatitis C patient to that of KHCV403 protein

Reactivity of UBNS5-1.2 protein with a serum taken from a hepatitis C patient was confirmed by employing the cell precipitates obtained in the above <Step 3> in the same manner as in <Step 4> of Example 1; and the result was compared to that of KHCV 403 protein which was confirmed by employing the yeast cell precipitate wherein KHCV 403 protein was produced according to Korean Laid-open Publication No. 93-12107 in the same manner as above. The results are shown in Fig. 19, wherein A is the result of SDS-PAGE and B is the result of Western blotting.

In Figs. 19 A and B, lane 1 shows the products of Saccharomyces cerevisiae DCO4-UB-KHCV 403(ATCC 74709); and lanes 2 shows the products of E. coli transformed with ptrpH-UB-NS5-1.2. From the result, it is confirmed that UBNS5-1.2 protein has much stronger reactivity with the serum taken from a hepatitis C patients than KHCV 403 protein.

<Step 6> Purification of UBNS5-1.2 protein

(6-1) Cell disruption and removal of soluble proteins

3g of E. coli cell precipitate obtained in <Step 3> was treated as in <Step 4>(4-1) of Example 3 to disrupt the cell and obtain insoluble precipitate therefrom.

(6-2) Washing of insoluble precipitate

The precipitate obtained in (6-1) was treated as in <Step 4>(4-2) of Example 3 to remove dissolved proteins and obtain insoluble precipitate.

(6-3) Dissolution of insoluble precipitate

The insoluble precipitate of (6-2) was suspended in 100mℓ of buffer 3(50mM Tris, pH 9.0, 1mM EDTA, 10mM ß-mercapto-ethanol) containing 8M urea. The suspension was stirred at room temperature for 1 hours and centrifuged to remove insoluble precipitate and obtain the supernatant.

(6-4) DEAE-Sepharose ion exchange chromatography

The supernatant obtained in (6-3) was passed through DEAE-Sepharose column(Pharmacia, 2.5cm x 3cm) equilibrated with the above buffer 3 at a flow rate of 2mℓ/min.; and same buffer was added to elute free proteins remains in column. Then, 300mℓ of buffer 3 having a concentration gradient of 0 to 0.3M NaCl was added at a flow rate of 8mℓ/min. to elute the bound proteins and collect the eluate by 4mℓ fractions. The protein fractions were subjected to 15% SDS-PAGE to collect the fractions comprising UBNS5-1.2 protein.

(6-5) S-300 gel filtration chromatography

The protein fractions collected in (6-4) was concentrated to a volume of 3mℓ with YM10 ultrafiltration membrane (Amicon, U.S.A.), and then, passed over S-300 resin column(Pharmacia, 1.2cm x 120cm) equilibrated with buffer 3 containing 8M urea at a flow rate of 10mℓ/hour. The eluted protein was collected by 0.5mℓ fractions and subjected to 15% SDS-PAGE to collect the fractions comprising highly pure UBNS5-1.2 protein.

(6-6) FPLC-phenyl-superose chromatography

The protein fractions collected in (6-5) were passed over YM10 ultrafiltration membrane (Amicon, U.S.A.) to concentrate to a volume of 4mℓ. The concentrate was dialyzed against PBS(10mM phosphate, pH 7.0, 15mM NaCl) using a dialysis membrane(Spectrum Medical Industries, Inc., M. W. cut off 6,000-8,000) to remove urea. To the solution was added sodium chloride to a final concentration of 1.5M. The resultant was passed over FPLC-phenyl-superose column(Pharmacia, HR 5/5, 0.5cm x 5cm) equilibrated with the

same buffer at a flow rate of 0.7mℓ/min.; and the same buffer was added to elute free proteins remained in column. Then, PBS containing a linear concentration gradient of 1.5M to 0 M sodium chloride was added to elute the bound proteins and collect the eluate by 0.7mℓ fractions. The protein fractions were subjected to 15% SDS-PAGE to collect the fractions comprising UBNS5-1.2 protein having a purity of at least 95%; and the antigenic specificity of the purified protein was confirmed by employing a western blotting analysis.

Example 6: Detection of anti-HCV antibodies with individual KHCV proteins and recombinant proteins of the present invention by employing ELISA(enzyme-linked immunosorbent assay) method

Each of KHCV CORE 14, KHCV 403 and KHCV NS5-1.2 protein was diluted with 50mM sodium borate buffer(pH 9.0) to a concentration of 0.3μg/mℓ. KHCV E2C and KHCV E1 protein were also diluted with the same buffer to a concentration of 0.2μg/mℓ and 0.1μg/mℓ, respectively. The diluted protein solutions were added to the wells of a microtiter plate(Dynatech, Immulon type 1 microtiter plate) in an amount of 200μℓ/well and incubated at 37°C for 2 hours.

The plate was washed once with PBS containing 0.05%(v/v) Tween-20(pH 7.4, hereinafter referred to as "washing solution"). PBS containing 0.1%(w/v) gelatin was added to the wells in an amount of 250μℓ/well; and the plate was incubated at 37°C for 1 hours to block the remaining protein adsorption sites so as to prevent any non-specific reactions which may occur later. The wells were washed twice with said washing solution and 190μℓ of PBS containing 0.25% gelatin, 1.0%(v/v), Triton X-100, 1mM EDTA and 0.02% Thimerosal was added to every well. Then, 10μℓ of serum samples taken from a HCV patient and a normal donor was added thereto and mixed gently for several seconds.

The wells which were reacted at 37°C for 1 hour were washed five times with the washing solution; and a solution comprising anti-human IgG antibody labelled with horseradish

peroxidase(HRP)(Bio-Rad Company, Richmond, CA 94804, U.S.A, 0.1mg protein/mℓ) which was diluted to a concentration of 1μg/mℓ with PBS containing 10% fetal bovine serum(v/v), 1% Ficoll(Sigma, v/v), 0.05% Tween-20 and 0.02% Thimerosal was added to the wells in an amount of 200μℓ/well.

The resultant was incubated at 37°C for 1 hour and washed 5 times with said washing solution. Thereafter, 200μℓ of substrate solution prepared by dissolving O-phenylene diamine dihydrochloric acid tablet(OPD tablet, Sigma) with 50mM citrate buffer to a concentration of 10mg/mℓ and adjusting to pH 5.5 by adding phosphate was added to each well and incubated at room temperature for 30 minutes in the dark. To the resultant was added 50μℓ of 4N sulfuric acid per each well to stop the color development; and O.D. of each well was determined at the wavelength of 492nm with Multiscan titertek(Flow Lab).

In addition, the same procedures as above were repeated by employing a mixed antigen solution comprising 250ng of KHCV CORE 518 protein, 125ng of KHCV NS4E1E2 protein and 125ng of KHCV NS5-1.2 protein per 1mℓ of 50mM sodium borate buffer(pH 9.0).

The results of the above procedures for detecting anti-KHCV antibodies by employing individual KHCV antigens and mixed antigens are shown in Table II below.

Table II. Detection of anti-HCV antibody with individual KHCV antigen and mixed antigens of the present invention

antigen Sample No.	KHCV CORE14	KHCV 897	KHCV 403	KHCV NS5-1.2	KHCV E1	KHCV E2	Mixed antigens
1	7.98	7.35	1.28	5.83	6.08	0.50	10.12
2	7.77	7.29	6.43	5.81	4.19	0.84	10.0
3	3.31	6.98	6.77	5.06	0.68	0.18	5.70
4	7.89	7.06	0.86	0.23	5.32	0.83	9.08
5	7.71	7.21	0.96	2.35	4.48	2.19	8.41
Positive control	7.53	7.35	7.44	5.87	5.95	8.89	8.83
Negative control	0.25	0.26	0.25	0.41	0.43	0.11	0.17

Note) 1. Each numerical value represents the absorbance/cutoff value

2. Positive control: HIV (-), HBV (-), HCV (+)
Negative control: HIV (-), HBV (-), HCV (-)

3. The serum samples including positive and negative controls were provided by Korean Red Cross Blood Center

In the above test, cutoff value was determined as follows:

509 HCV-negative and 76 HCV-positive serum samples confirmed with RIBA II diagnostic kit(Ortho Diagnostic Systems, U.S.A.) by employing immunoblotting assay method were tested according to the above diagnostic process to obtain the result shown in Table III.

Table III

	Number of samples	Average of OD_{490}	Standard deviation
negative sample	509	0.098	0.095
positive sample	76	1.791	0.809

Then, cutoff value was calculated by employing the

following general equation for adjusting cutoff value:

Cutoff value = average of OD_{490} of negative samples + 3 x standard deviation of OD_{490} of negative samples

Accordingly, a cutoff value of 0.4 was obtained by reference to the calculated cutoff value of 0.383 and the distribution of OD_{490} of negative samples.

As can be seen from the above Table II, individual antigen, as well as the mixed antigen may be used as a diagnostic agent for detecting anti-HCV antibody; however the mixed antigen shows more sensitive and district results between negative and positive samples.

Example 7: Detection of anti-KHCV antibodies by employing individual recombinant protein comprising KHCV epitopes and mixed KHCV antigens

Each of KHCV E1E2 and KHCV NS4E1E2 proteins was diluted with 50mM sodium borate buffer(pH 9.0) to a concentration of 0.2μg/mℓ; and an antigen solution comprising 250ng of KHCV CORE 518 protein, 125ng of KHCV NS4E1E2 protein and 125ng of NS5-1.2 protein per 1mℓ of 50mM sodium borate buffer(pH 9.0) was prepared. The diluted protein solutions were added to the wells of a microtiter plate(Dynatech, Immulon type 1 microtiter plate) in an amount of 200μℓ/well and the same procedures as in the Example 6 were repeated to obtain the result shown in Table IV below.

Table IV. Detection of anti-HCV antibody with mixed antigens

antigen / Sample No.	KHCV E1E2	KHCV NS4E1E2	mixed antigens
1	0.222	0.233	0.180
2	7.834	8.10	8.301
3	4.554	7.067	8.14
4	0.255	0.233	0.190
5	0.573	7.700	7.470
6	0.255	0.300	0.217
7	0.478	0.700	0.640
8	0.446	0.533	0.314
9	0.032	0.067	0.090
10	0.510	0.433	0.303
11	0.478	0.460	0.467
12	0.350	0.667	0.737
13	7.548	7.900	8.755
14	7.675	8.133	8.502
15	0.573	0.060	0.294
16	0.669	0.567	0.377
17	0.414	0.433	0.257
18	0.382	0.460	0.304
Positive control	7.197	7.510	8.065
Negative control	0.280	0.250	0.193

Note) 1. Each numerical value represents the absorbance/cutoff value

2. Positive control: HIV (-), HBV (-), HCV (+)
Negative control: HIV (-), HBV (-), HCV (-)

As can be seen from the above Table IV, KHCV NS4E1E2 protein was more effective than KHCV E1E2 protein in detecting anti-KHCV antibodies from the serum taken from a hepatitis C patients, and the mixed antigens exhibited higher effectiveness than KHCV NS4E1E2 protein itself owing to the additive effect of other antigens included in the mixture.

In addition, each of KHCV COREEPI, KHCV 518 and KHCV CORE518 protein was diluted with 50mM sodium borate buffer(pH 9.0) to a concentration of 125ng/mℓ; the antigen solution was added to the wells of a microtiter plate(Dynatech, Immulon type 1 microtiter plate) in an amount of 200μℓ/well; and the same procedures as in the Example 6 were repeated by employing 136 sera taken from hepatitis C patients(which were obtained from Hyundai Central Hospital, Seoul, Korea). The result obtained in the above procedure is shown in Table V comparatively with the result obtained by PCR method.

Table V

KHCV COREEPI	KHCV 518	KHCV CORE518	PCR
119(+)	83(+)	123(+)	123(+)
13(-)	11(-)	12(-)	13(-)

As can be seen from the above Table V, the diagnostic results obtained by employing the KHCV COREEPI protein and KHCV 518 protein were less sensitive than the diagnostic result obtained by employing the KHCV CORE518 protein which comprises epitopes of the above two KHCV proteins.

Example 8: Comparison of diagnostic methods of prior art and the present invention

The same procedures as in the Example 6 were repeated by employing samples obtained periodically from human HCV seroconversional panels after blood transfusion(Serological Inc., 780 Park North Blud, Clarkston, GA 30021, USA) to detect anti-HCV antibodies therein; and the result is shown in Table VI below, comparatively with the results obtained by employing Ortho 1st generation and Abbott 1st generation HCV diagnostic Kits.

Table VI. Detection of anti-HCV antibodies in seroconversional samples

Days after blood trans-fusion			HCV diagnostic kit			Confirm test
	ALT mU/mℓ	AST mU/mℓ	Ortho first genera tion	Abbott first genera-tion	mixed antigens	RIBAII
1	11	-	0.29	0.263	0.49	-
11	24	-	0.33	0.253	0.44	-
15	36	-	0.29	0.267	0.45	-
18	36	-	0.27	0.255	0.50	-
22	40	-	0.28	0.251	0.52	-
25	24	-	0.16	0.220	0.45	-
29	32	-	0.16	0.265	0.44	-
32	27	27	0.20	0.259	0.45	-
36	32	-	0.23	0.270	0.50	-
39	78	-	0.14	0.275	0.47	-
43	180	121	0.23	0.303	0.40	-
74	401	352	0.80	0.495	5.32	+
114	72	70	6.21	4.356	6.73	+
127	42	37	6.21	4.356	7.71	+
141	27	24	6.21	4.356	9.24	+
155	68	69	6.21	4.356	9.11	+
175	78	97	6.21	4.356	7.99	-
238	41	39	6.21	4.356	10.15	+
270	119	102	6.21	4.356	8.77	+
297	49	35	6.21	4.356	8.56	+
365	157	128	6.21	4.356	9.72	+
399	46	19	6.21	4.356	9.45	+

Note) 1. Each numerical value represents the absorbance/cutoff value

2. The activities of alanine aminotransferase(ALT) and aspartate aminotransferase(AST) are normally 0 - 50mU/ml.

3. Ortho 1st generation HCV diagnostic kit was commercially available from Ortho Diagnostic Systems, U.S.A.

4. Abbott 1st generation HCV diagnostic kit was commercially available from Abbott Lab., U.S.A.

5. RIBA II HCV Test System was commercially available from Ortho Diagnostic Systems, U.S.A.

The above result reveals that the diagnostic kit of the present invention can detect anti-HCV antibodies more earlier (about 5-6 weeks earlier) than the other 1st generation diagnostic kits, and similar to RIBA II kit even though the diagnostic method of the present invention employs ELISA method which is more convenient than the immunoblotting assay adopted by RIBA II kit.

Example 9: Accuracy of diagnosis

To demonstrate the accuracy of the result of the present diagnosis, 18 serum samples which had been diagnosed as positive by using the diagnostic kit for hepatitis C manufactured and sold by Ortho Diagnostic Systems were diagnosed again with the diagnostic kit of the present invention according to the process of Example 7; and also with the Ortho 2nd generation immunoblotting kit for diagnosing hepatitis C(Ortho Diagnostic Systems, U.S.A., Product Code 933491), which is recommended as a confirmation assay(Van der poel, C. L. et al., Lancet 337, 317-319(1991)), in accordance with the manufacturer's instruction. These results are summarized in Table VII, which show that the diagnostic kit of the present invention has a lower false positive than Ortho's diagnostic kit for hepatitis C.

Table VII. Comparison of diagnostic result according to the present invention to that using Ortho 2nd generation diagnostic kit.

Sample No.	Antigens of Ortho second generation recombinant immunoblotting kit						Mixed antigens of the present invention
	5-1-1	C100-3	C33C	C22-3	SOD	judgement	
1	+/-	+/-	-	-	-	-	0.180
2	+4	+4	+4	+4	-	+	8.301
3	+1	+4	+4	+4	-	+	8.141
4	-	-	-	-	-	-	0.190
5	+1	+4	+4	+4	-	+	7.470
6	-	-	-	-	-	-	0.217
7	-	-	-	-	-	-	0.640
8	-	-	-	-	-	-	0.314
9	-	-	-	-	-	-	0.090
10	-	-	-	-	-	-	0.303
11	-	-	-	-	-	-	0.467
12	-	-	-	-	-	-	0.737
13	+2	+1	+3	+4	-	+	8.755
14	+/-	+/-	+4	+4	-	+	8.502
15	-	-	-	-	-	-	0.294
16	-	-	-	-	-	-	0.377
17	-	-	-	-	-	-	0.257
18	-	+/-	-	-	-	-	0.304

Note) If a sample found to have more than 1 in at least two antigens except the SOD control antigen, then it was judged to be positive.

Example 10. Specificity of the diagnostic kit

To demonstrate the specificity and sensitivity of the present diagnostic agent, 94 serum samples which had been diagnosed as positive by using any of the diagnostic kit for hepatitis C selected from a group consisting of Lucky I(which is the diagnostic kit developed by Lucky Limited and disclosed in Korean Patent Publication No. 93-683), Abbott 2nd generation diagnostic kit(Abbott II) and UBI(UBI Co., U.S.) were diagnosed again with the diagnostic kit of the present invention according to the process of Example 7. The results are summarized in Table VIII below.

As can be seen from Table VIII, all of the diagnostic kits exhibited similar sensitivities, and the diagnostic kit of the present invention(Lucky II) has higher specificity than other diagnostic kits for hepatitis C.

Table VIII

Confirm test (RIBA II)		Abbott II	UBI	Lucky I	Lucky II
Positive 51	+	49	50	48	49
	-	2	1	3	2
Indeterminate 8	+	6	4	5	2
	-	2	4	3	6
Negative 35	+	6	19	10	2
	-	29	16	25	33

* Judgement of the test results as true positive, true negative, false positive or false negative were carried out according to the result of RIBA II confirm test.

In addition, 278 serum samples were selected at random from the serum samples diagnosed as negative by using any of the above four diagnostic kits(Abbott II, UBI, Lucky I and Lucky II) and the average absorbance(OD_{492}) of the selected samples were calculated. The same procedures were repeated to obtain the average absorbance of 94 serum samples diagnosed as positive by using any of the diagnostic kit

selected from a group consisting of Lucky I, Abbott II and UBI.

The average absorbance of positive samples(AVG of P) and average absorbance of negative samples(AVG of N) were divided respectively by average cutoff value to obtain average signal/cutoff(S/C) values of positive samples and negative samples. The results are shown in Table IX, below. As can be seen from Table IX, Lucky II and UBI have the largest differencees between the S/C values of positive and negative samples, which represents that they give clearer signal than others. However, the high S/C value of UBI is not significant, because it depends on some extent on the low cutoff value and, consequently, high false-positive rate may be resulted.

Table IX

	Abbott II	UBI	Lucky I	Lucky II
AVG of P	1.805	1.366	1.344	2.053
AVG of N	0.096	0.021	0.058	0.037
Cutoff	0.52	0.25	0.44	0.42
S/C of P	3.471	5.464	3.055	4.888
S/C of N	0.185	0.084	0.132	0.088

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes which may be apparent to those skilled in the art to which the invention pertains may be made and also fall within the scope of the invention as defined by the claims that follow.

What is claimed is:

1. An antigenic protein of hepatitis C virus selected from the group consisting of KHCV COREEPI, KHCV 518, KHCV NS4E, KHCV E1G, KHCV E2A, KHCV E2E and KHCV NS5-1.2 having the amino acid sequences of:

KHCV COREEPI:

MetSerThrAsnProLysProGlnArgLysThrLysArgAsnThrAsnArgArgProGln

AspIleLysPheProGlyGlyGlyGlnIleValGlyGlyValTyrLeuLeuProArgArg

GlyProArgLeuGlyValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly

ArgArgGlnProIleProLysAlaArgArgProGluGlyArgAlaTrpAlaGlnProGly

TyrProTrpProLeuTyrGlyAsnGluGlyLeuGlyTrpAlaGlyTrpLeuLeuSerPro

ArgGly ;

KHCV 518:

IleThrThrGlyAlaProIleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGly

GlySerGlyGlyAlaTyrAspIleIleMetCysAspGluCysHisSerThrAspSerThr

ThrIleTyrGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGlyAlaArgLeuVal

ValLeuSerThrAlaThrProProGlySerValThrValProHisLeuAsnIleGluGlu

ValAlaLeuSerAsnThrGlyGluIleProPheTyrGlyLysAlaIleProIleGluAla

IleLysGlyGlyArgHisLeuIlePheCysHisSerLysLysLysCysAspGluLeuAla
AlaLysLeuSerGlyLeuGlyLeuAsnAlaValAlaTyrTyrArgGlyLeuAspValSer
ValIleProThrSerGlyAspValValValValAlaThrAspAlaLeuMetThrGlyPhe
ThrGlyAspPheAspSerValIleAspCysAsnThrCys ;

KHCV NS4E:

IleIleProAspArgGluValLeuTyrGlnGluPheAspGluMetGluGluCysAlaSer
HisLeuProTyrPheGluGlnGlyMetGlnLeuAlaGluGlnPheLysGlnLysAlaLeu
GlyLeu ;

KHCV E1G:

ValSerGlnLeuPheThrPheSerProArgArgHisGluThrValGlnAspCysAsnCys
SerIleTyrProGlyArgValSerGlyHisArgMetAlaTrpAspMetMetMetAsnTrp
SerProThrThrAlaLeuValValSerGlnLeuLeuArgIleProGlnAlaValValAsp
MetValThrGlySerHisTrpGlyIleLeuAlaGlyLeuAlaTyrTyrSerMetValGly
AsnTrpAlaLysValLeuIleAlaMetLeuLeuPheAlaGlyValAspGlyThrThrHis
ValThrGly ;

KHCV E2A:

GlyAlaGlnGlyArgAlaAlaSerSerLeuThrSerLeuPheSerProGlyProValGln

HisLeuGlnLeuIleAsnThrAsnGlySerTrpHisIleAsnArgThrAlaLeuSerCys

AsnAspSerLeuAsnThrGlyPheValAlaAlaLeuPheTyrLysTyrArgPheAsnAla

SerGlyCysProGluArgLeuAlaThrCysArgProIleAspThrPheAlaGlnGlyTrp ;

KHCV E2E:

ThrArgGlyGluArgCysAspLeuGluAspArgAspArgSerGluLeuSerProLeuLeu

LeuSerThrThrGluTrpGlnValLeuProCysSerPheThrThrLeuProAlaLeuSer

ThrGlyLeuIleHisLeuHisGlnAsnIleValAspIleGlnTyrLeuTyrGlyIleGly

SerAlaValValSerPheAlaIleLysTrpGluTyrIleValLeuLeuPheLeuLeuLeu

AlaAspAla ; and

KHCV NS5-1.2:

ThrGlyMetThrThrAspAsnValLysCysProCysGlnValProAlaProGluPhePhe

ThrGluValAspGlyValArgLeuHisArgTyrAlaProAlaCysArgProLeuLeuArg

GluGluValValPheGlnValGlyLeuHisGlnTyrLeuValGlySerGlnLeuProCys

GluProGluProAspValAlaValLeuThrSerMetLeuThrAspProSerHisIleThr

AlaGluThrAlaLysArgArgLeuAlaArgGlySerProProSerLeuAlaSerSerSer

AlaSerGlnLeuSerAlaProSerLeuLysAlaThrCysThrThrHisHisAspSerPro

AspAlaAspLeuIleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsnIleThr

ArgValGluSerGluAsnLysValValIleLeuAspSerPheAspProLeuArgAlaGlu

AspAspGluGlyGluIleSerValProAlaGluIleLeuArgLysSerArgLysPhePro

ProAlaLeuProIleTrpAlaProProAspTyrAsnProProLeuLeuGluSerTrpLys

AspProAspTyrValProProValValHisGlyCysProLeuProProThrLysAlaPro

ProIleProProProArgArgLysArgThrValValLeuThrGluSerThrValSerSer

AlaLeuAlaGluLeuAlaThrLysThrPheGlySerSerGlySerSerAlaIleAspSer

GlyThrAlaThrAlaProProAspGlnAlaSerGlyAspGlyAspArgGluSerAspVal

GluSerPheSerSerMetProProLeuGluGlyGluProGlyAspProAspLeuSerAsp

GlySerTrpSerThrValSerGluGluAlaSerGluAspValValCysCysSerMetSer

TyrThrTrpThrGlyAlaLeuIleThrProCysAlaAlaGluGluSerLysLeuProIle

AsnProLeuSerAsnSerLeuLeuArgHisHisAsnMetValTyrAlaThrThrSerArg

SerAlaGlyLeuArgGlnLysLysValThrPheAspArgLeuGlnValLeuAspAspHis

TyrArgAspValLeuLysGluMetLysAlaLysAla.

2. A recombinant protein comprising one or more antigenic proteins of hepatitis C virus of claim 1.

3. The recombinant protein of claim 2 which comprises KHCV COREEPI and KHCV 518.

4. The recombinant protein of claim 2 which comprises KHCV E1G, KHCV E2A and KHCV E2E.

5. The recombinant protein of claim 4 which further comprises KHCV NS4E.

6. The recombinant protein of any one of claims 2 to 5 which further comprises ubiquitin.

7. A DNA fragment comprising a nucleotide sequence encoding the hepatitis C antigen protein of claim 1.

8. The DNA fragment of claim 7 comprising the nucleotide sequences encoding KHCV COREEPI and KHCV 518.

9. The DNA fragment of claim 7 comprising the nucleotide sequences encoding KHCV NS4E and KHCV 518.

10. The DNA fragment of claim 7 comprising the nucleotide sequences encoding KHCV NS4E, KHCV E1G, KHCV E2A and KHCV E2E.

11. The DNA fragment of claim 7 comprising the nucleotide sequences encoding KHCV E1G, KHCV E2A and KHCV E2E.

12. An expression vector comprising the DNA fragment of any one of claims 7 to 11.

13. The expression vector of claim 12 which is selected from the group consisting of ptrpH-UB-CORE 518, ptrpH-UB-NS4E1E2, ptrpH-UB-NS5-1.2 and ptrpH-UB-E1E2.

14. An E. coli cell transformed with the expression vector of claim 12 or 13.

15. A process for preparing an antigenic protein of hepatitis C virus comprising culturing the E. coli transformant of claim 14 and recovering the antigenic protein from the culture.

16. The process of claim 15 wherein KHCV 518 is prepared by employing the E. coli cell transformed with ptrpH-UB-CORE 518.

17. The process of claim 15 wherein KHCV NS4E1E2 is prepared by employing the E. coli cell transformed with ptrpH-UB-NS4E1E2.

18. The process of claim 15 wherein KHCV NS5-1.2 is prepared by employing the E. coli cell transformed with ptrpH-UB-NS5-1.2.

19. A diagnostic agent for detecting the antibody directed against a hepatitis C viral antigen in a putative sample comprising one or more antigenic proteins selected from the group consisting of KHCV CORE-518, KHCV NS4E1E2 and KHCV NS5-1.2.

20. A diagnostic method for detecting an antibody directed against a hepatitis C viral antigen in a putative sample by employing the diagnostic agent of claim 19.

21. The diagnostic method of claim 20 wherein the diagnosis is carried out by employing enzyme-linked immunosorbent assay(ELISA).

Fig. 1

KHCV COREEPI

```
                .         .        30         .         .        60
ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCCACAG
MetSerThrAsnProLysProGlnArgLysThrLysArgAsnThrAsnArgArgProGln

                .         .        90         .         .       120
GATATTAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACTTGTTGCCGCGCAGG
AspIleLysPheProGlyGlyGlyGlnIleValGlyGlyValTyrLeuLeuProArgArg

                .         .       150         .         .       180
GGCCCCAGGTTGGGTGTGCGCGCGACTAGGAAGACTTCCGAGCGGTCGCAACCTCGTGGA
GlyProArgLeuGlyValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly

                .         .       210         .         .       240
AGGCGACAGCCTATCCCCAAGGCTCGCCGGCCCGAGGGCAGGGCCTGGGCTCAGCCCGGG
ArgArgGlnProIleProLysAlaArgArgProGluGlyArgAlaTrpAlaGlnProGly

                .         .       270         .         .       300
TACCCTTGGCCCCTCTATGGCAATGAGGGCTTGGGGTGGGCAGGATGGCTCCTGTCACCC
TyrProTrpProLeuTyrGlyAsnGluGlyLeuGlyTrpAlaGlyTrpLeuLeuSerPro

CGCGGC
ArgGly
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Fig. 1 continued

KHCV 518

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1   ATCACCACAGGTGCCCCTATCACATACTCCACCTATGGCAAGTTCCTTGCCGACGGTGGC
    IleThrThrGlyAlaProIleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGly

61  GGCTCCGGGGGCGCCTATGACATCATAATGTGTGATGAGTGCCACTCAACTGACTCGACT
    GlySerGlyGlyAlaTyrAspIleIleMetCysAspGluCysHisSerThrAspSerThr

121 ACCATTTATGGCATCGGCACAGTCCTGGACCAAGCGGAGACGGCTGGAGCGCGGCTCGTG
    ThrIleTyrGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGlyAlaArgLeuVal

181 GTGCTCTCCACCGCTACGCCTCCGGGATCGGTCACCGTGCCACACCTCAATATCGAGGAG
    ValLeuSerThrAlaThrProProGlySerValThrValProHisLeuAsnIleGluGlu

241 GTGGCCCTGTCTAATACTGGAGAGATCCCCTTCTACGGCAAAGCCATTCCCATCGAGGCT
    ValAlaLeuSerAsnThrGlyGluIleProPheTyrGlyLysAlaIleProIleGluAla

301 ATCAAGGGGGGAAGGCATCTCATTTTCTGCCATTCCAAGAAGAAGTGTGACGAACTCGCC
    IleLysGlyGlyArgHisLeuIlePheCysHisSerLysLysLysCysAspGluLeuAla

361 GCAAAGCTGTCAGGCCTCGGACTCAATGCCGTAGCGTATTACCGGGGTCTTGACGTGTCC
    AlaLysLeuSerGlyLeuGlyLeuAsnAlaValAlaTyrTyrArgGlyLeuAspValSer

421 GTCATACCGACCAGCGGAGACGTTGTTGTCGTGGCGACGGACGCTCTAATGACGGGCTTT
    ValIleProThrSerGlyAspValValValValAlaThrAspAlaLeuMetThrGlyPhe

481 ACCGGCGACTTTGACTCAGTGATCGACTGTAATACGTGT
    ThrGlyAspPheAspSerValIleAspCysAsnThrCys
```

Fig. 2

KHCV NS4E

```
  1 ATCATCCCCGATAGGGAAGTTCTCTACCAGGAGTTCGACGAGATGGAGGAGTGTGCCTCA
    IleIleProAspArgGluValLeuTyrGlnGluPheAspGluMetGluGluCysAlaSer

 61 CACCTCCCTTACTTCGAACAGGGAATGCAGCTCGCCGAGCAATTCAAACAGAAGGCGCTC
    HisLeuProTyrPheGluGlnGlyMetGlnLeuAlaGluGlnPheLysGlnLysAlaLeu

121 GGGTTG
    GlyLeu
```

KHCV E1G

```
  1 GTTTCCCAGCTGTTCACCTTTTCGCCTCGCCGGCATGAGACGGTACAGGACTGCAACTGC
    ValSerGlnLeuPheThrPheSerProArgArgHisGluThrValGlnAspCysAsnCys

 61 TCAATCTATCCCGGCCGCGTATCAGGTCACCGCATGGCCTGGGATATGATGATGAACTGG
    SerIleTyrProGlyArgValSerGlyHisArgMetAlaTrpAspMetMetMetAsnTrp

121 TCGCCTACAACAGCCCTAGTGGTATCGCAGCTACTCCGGATCCCACAAGCTGTCGTGGAC
    SerProThrThrAlaLeuValValSerGlnLeuLeuArgIleProGlnAlaValValAsp

181 ATGGTGACAGGGTCCCACTGGGGAATCCTGGCGGGCCTTGCCTACTATTCCATGGTGGGG
    MetValThrGlySerHisTrpGlyIleLeuAlaGlyLeuAlaTyrTyrSerMetValGly

241 AACTGGGCTAAGGTCTTAATTGCGATGCTACTCTTTGCCGGCGTTGACGGAACCACCCAC
    AsnTrpAlaLysValLeuIleAlaMetLeuLeuPheAlaGlyValAspGlyThrThrHis

301 GTGACAGGG
    ValThrGly
```

Fig. 2 continued

KHCV E2A

```
  1 GGGGCGCAAGGTCGGGCCGCTAGCTCGCTAACGTCCCTCTTTAGCCCTGGGCCGGTTCAG
    GlyAlaGlnGlyArgAlaAlaSerSerLeuThrSerLeuPheSerProGlyProValGln

 61 CACCTCCAGCTCATAAACACCAACGGCAGCTGGCATATCAACAGGACCGCCCTGAGCTGC
    HisLeuGlnLeuIleAsnThrAsnGlySerTrpHisIleAsnArgThrAlaLeuSerCys

121 AATGACTCCCTCAACACTGGGTTTGTTGCCGCGCTGTTCTACAAATACAGGTTCAACGCG
    AsnAspSerLeuAsnThrGlyPheValAlaAlaLeuPheTyrLysTyrArgPheAsnAla

181 TCCGGGTGCCCCGGAGCGCTTGGCCACGTGCCGCCCCATTGATACATTCGCGCAGGGGTGG
    SerGlyCysProGluArgLeuAlaThrCysArgProIleAspThrPheAlaGlnGlyTrp
```

KHCV E2E

```
  1 ACTCGGGGAGAGCGTTGTGACCTGGAGGACAGGGATAGGTCAGAGCTTAGCCCGCTGCTG
    ThrArgGlyGluArgCysAspLeuGluAspArgAspArgSerGluLeuSerProLeuLeu

 61 CTGTCTACAACAGAGTGGCAGGTACTGCCCTGTTCCTTCACAACCCTACCGGCTCTGTCC
    LeuSerThrThrGluTrpGlnValLeuProCysSerPheThrThrLeuProAlaLeuSer

121 ACTGGTTTGATTCATCTCCATCAGAACATCGTGGACATACAATACCTGTACGGTATAGGG
    ThrGlyLeuIleHisLeuHisGlnAsnIleValAspIleGlnTyrLeuTyrGlyIleGly

181 TCGGCGGTTGTCTCCTTTGCGATCAAATGGGAGTATATTGTGCTGCTCTTCCTTCTTCTG
    SerAlaValValSerPheAlaIleLysTrpGluTyrIleValLeuLeuPheLeuLeuLeu

241 GCGGACGCG
    AlaAspAla
```

Fig. 3

KHCV NS5-1.2

```
   1 ACGGGCATGACCACTGACAACGTGAAGTGTCCATGCCAGGTTCCGGCCCCCGAATTCTTC
     ThrGlyMetThrThrAspAsnValLysCysProCysGlnValProAlaProGluPhePhe

  61 ACGGAGGTGGATGGAGTGCGGTTGCACAGGTACGCTCCGGCGTGCAGACCTCTCCTACGG
     ThrGluValAspGlyValArgLeuHisArgTyrAlaProAlaCysArgProLeuLeuArg

 121 GAGGAGGTCGTATTCCAGGTCGGGCTCCACCAGTACCTGGTCGGGTCACAGCTCCCATGC
     GluGluValValPheGlnValGlyLeuHisGlnTyrLeuValGlySerGlnLeuProCys

 181 GAGCCCGAACCGGATGTAGCAGTGCTCACTTCCATGCTCACTGACCCCTCCCACATTACA
     GluProGluProAspValAlaValLeuThrSerMetLeuThrAspProSerHisIleThr

 241 GCAGAGACGGCTAAGCGTAGGCTGGCCAGGGGGTCTCCCCCCTCCTTGGCCAGCTCTTCA
     AlaGluThrAlaLysArgArgLeuAlaArgGlySerProProSerLeuAlaSerSerSer

 301 GCTAGCCAGTTGTCTGCGCCTTCCTTGAAGGCGACATGCACTACCCATCATGACTCCCCG
     AlaSerGlnLeuSerAlaProSerLeuLysAlaThrCysThrThrHisHisAspSerPro

 361 GACGCTGACCTCATTGAGGCCAACCTCTTGTGGCGGCAAGAGATGGGCGGGAACATCACC
     AspAlaAspLeuIleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsnIleThr

 421 CGCGTGGAGTCAGAGAATAAGGTGGTAATCCTGGACTCTTTCGACCCGCTCCGAGCGGAG
     ArgValGluSerGluAsnLysValValIleLeuAspSerPheAspProLeuArgAlaGlu

 481 GATGATGAGGGGGAAATATCCGTTCCGGCGGAGATCCTGCGGAAATCCAGGAAATTCCCC
     AspAspGluGlyGluIleSerValProAlaGluIleLeuArgLysSerArgLysPhePro

 541 CCAGCGCTGCCCATATGGGCGCCGCCGGATTACAACCCTCCGCTGCTAGAGTCCTGGAAG
     ProAlaLeuProIleTrpAlaProProAspTyrAsnProProLeuLeuGluSerTrpLys
```

Fig. 3 continued

```
601 GACCCGGACTACGTTCCTCCGGTGGTACACGGGTGCCCGTTGCCGCCCACCAAGGCCCCT
    AspProAspTyrValProProValValHisGlyCysProLeuProProThrLysAlaPro

661 CCAATACCACCTCCACGGAGGAAGAGGACGGTTGTCCTGACAGAATCCACCGTGTCTTCT
    ProIleProProProArgArgLysArgThrValValLeuThrGluSerThrValSerSer

721 GCCTTGGCGGAGCTCGCTACTAAGACCTTCGGCAGCTCCGGATCGTCGGCCATCGACAGC
    AlaLeuAlaGluLeuAlaThrLysThrPheGlySerSerGlySerSerAlaIleAspSer

781 GGTACGGCGACCGCCCCTCCTGACCAAGCCTCCGGTGACGGCGACAGAGAGTCCGACGTT
    GlyThrAlaThrAlaProProAspGlnAlaSerGlyAspGlyAspArgGluSerAspVal

841 GAGTCGTTCTCCTCCATGCCCCCCCTTGAGGGAGAGCCGGGGGACCCCGATCTCAGCGAC
    GluSerPheSerSerMetProProLeuGluGlyGluProGlyAspProAspLeuSerAsp

901 GGATCTTGGTCCACCGTGAGCGAGGAGGCTAGTGAGGACGTCGTCTGCTGTTCGATGTCC
    GlySerTrpSerThrValSerGluGluAlaSerGluAspValValCysCysSerMetSer

961 TACACATGGACAGGCGCCCTGATCACGCCATGCGCTGCGGAGGAAAGCAAGTTGCCCATC
    TyrThrTrpThrGlyAlaLeuIleThrProCysAlaAlaGluGluSerLysLeuProIle

1021 AACCCGTTGAGCAATTCTTTGCTACGTCACCACAACATGGTCTATGCTACAACATCCCGC
     AsnProLeuSerAsnSerLeuLeuArgHisHisAsnMetValTyrAlaThrThrSerArg

1081 AGCGCAGGCCTGCGGCAGAAGAAGGTCACCTTTGACAGACTGCAAGTCCTGGACGACCAC
     SerAlaGlyLeuArgGlnLysLysValThrPheAspArgLeuGlnValLeuAspAspHis

1141 TACCGGGACGTGCTTAAGGAGATGAAGGCGAAGGCG
     TyrArgAspValLeuLysGluMetLysAlaLysAla
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Fig. 4

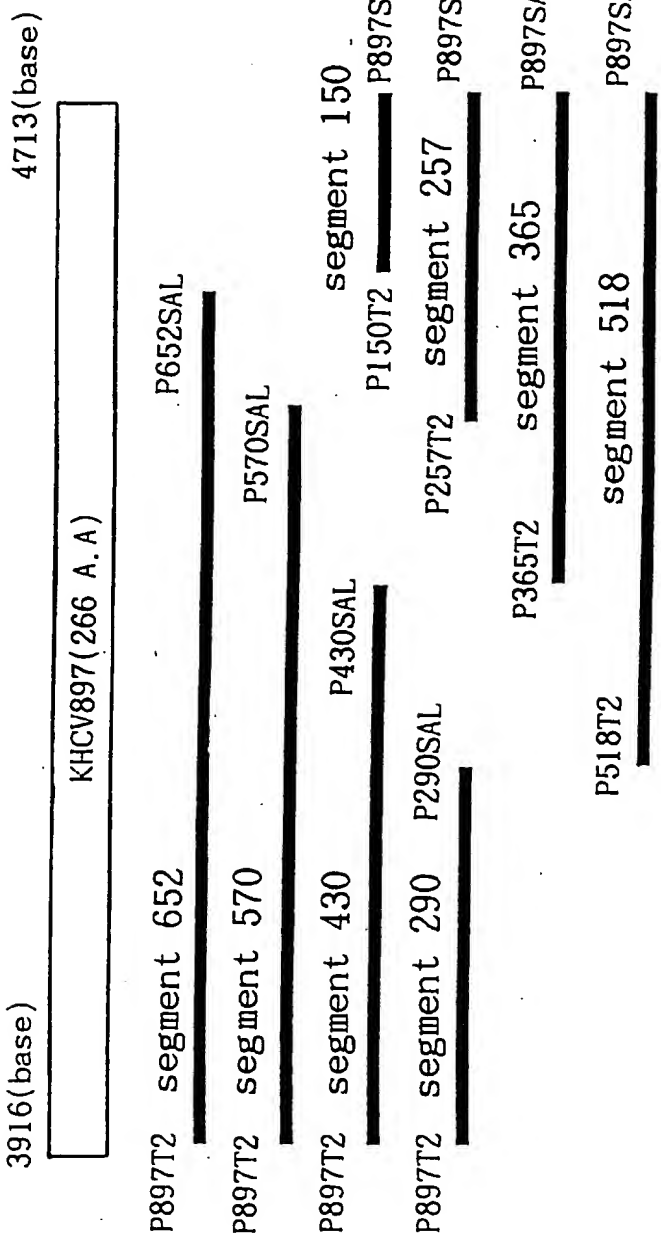


Fig. 5

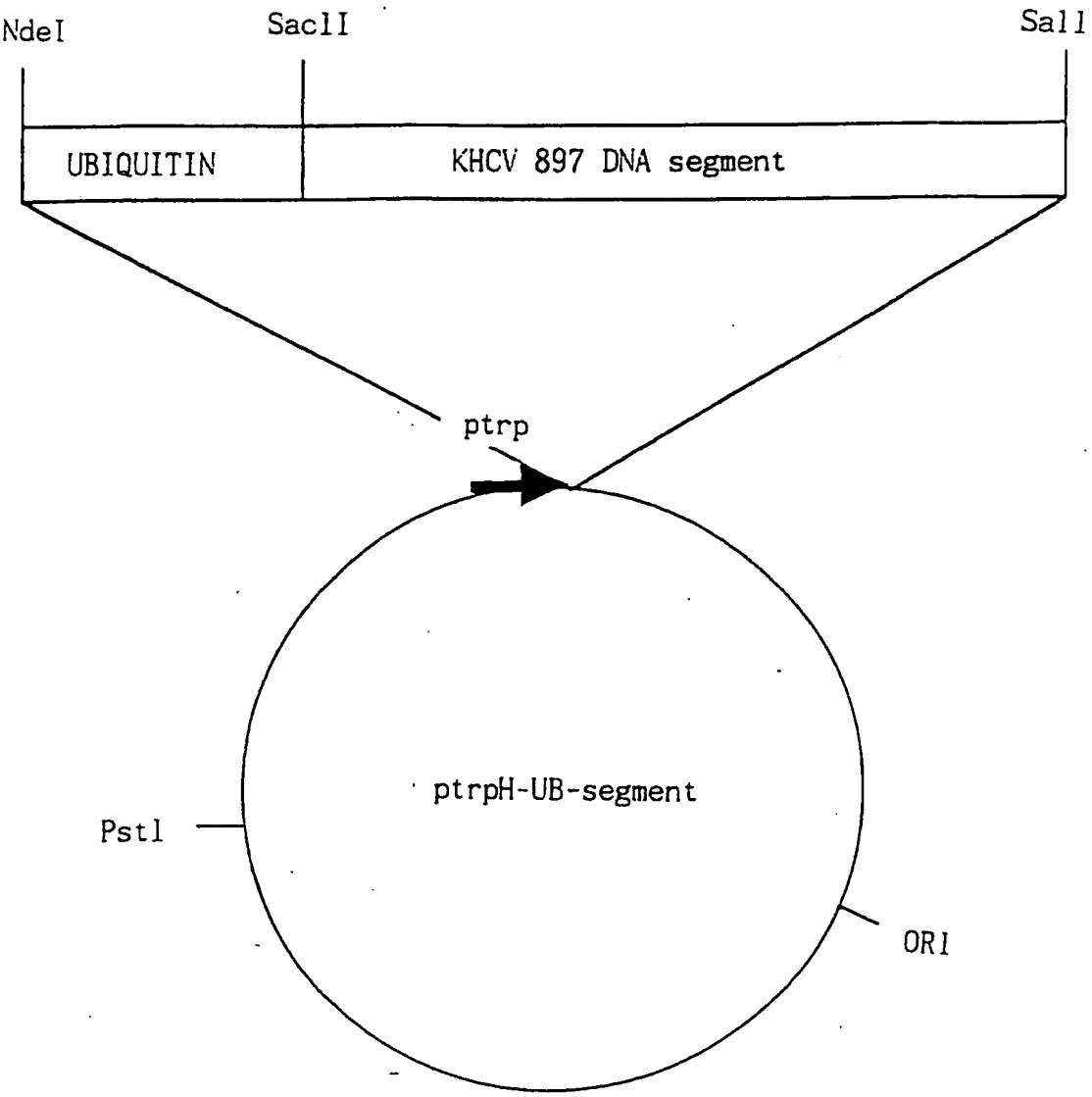

NdeI
SacII
SalI
UBIQUITIN
KHCV 897 DNA segment
ptrp
ptrpH-UB-segment
PstI
ORI

Fig. 6

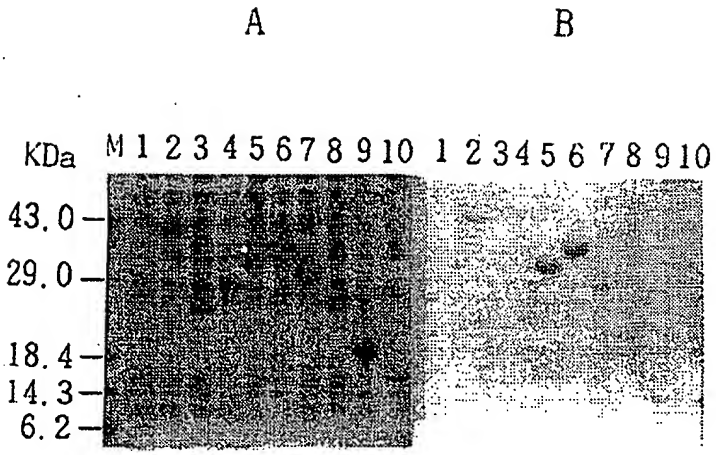
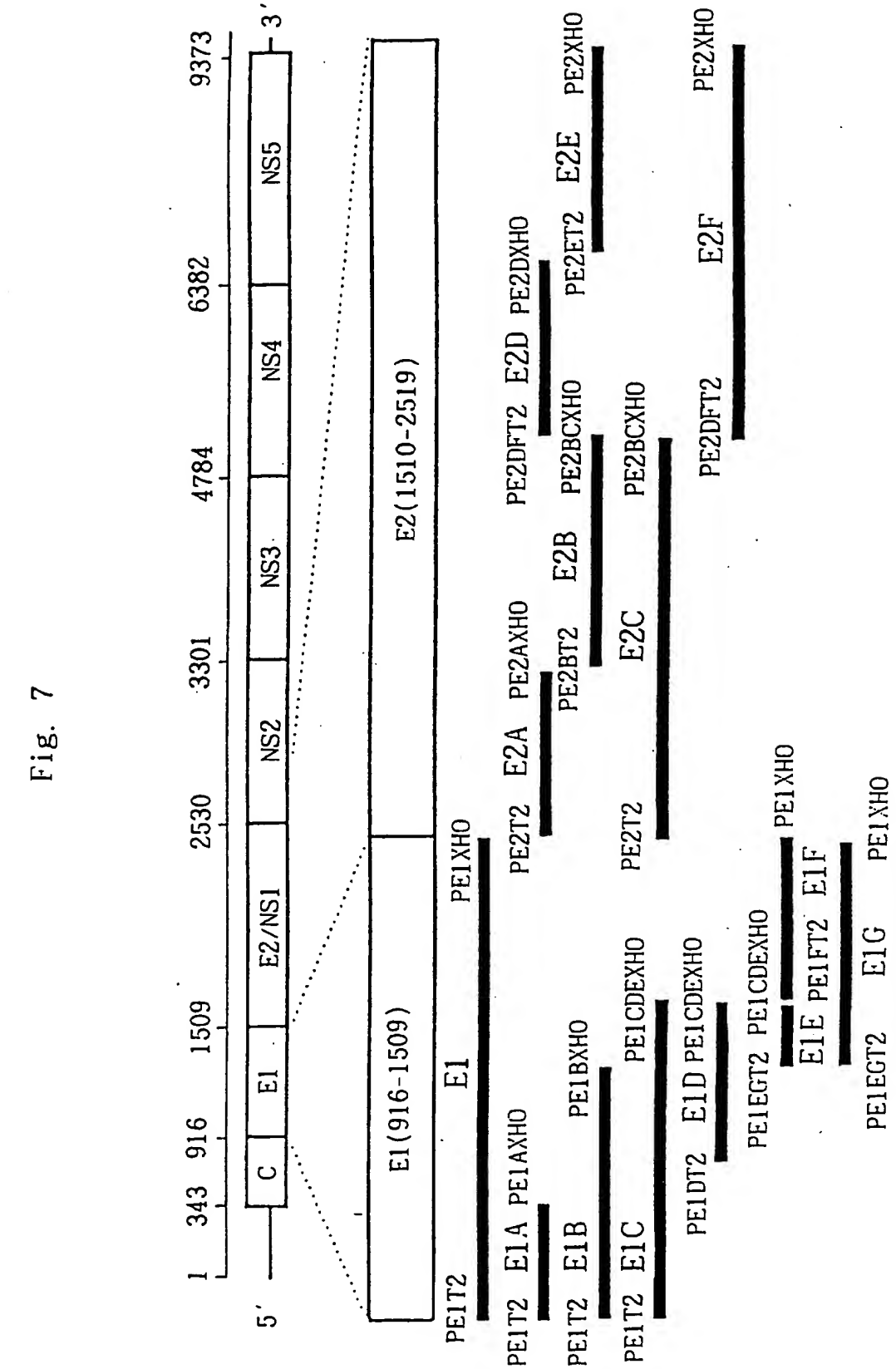
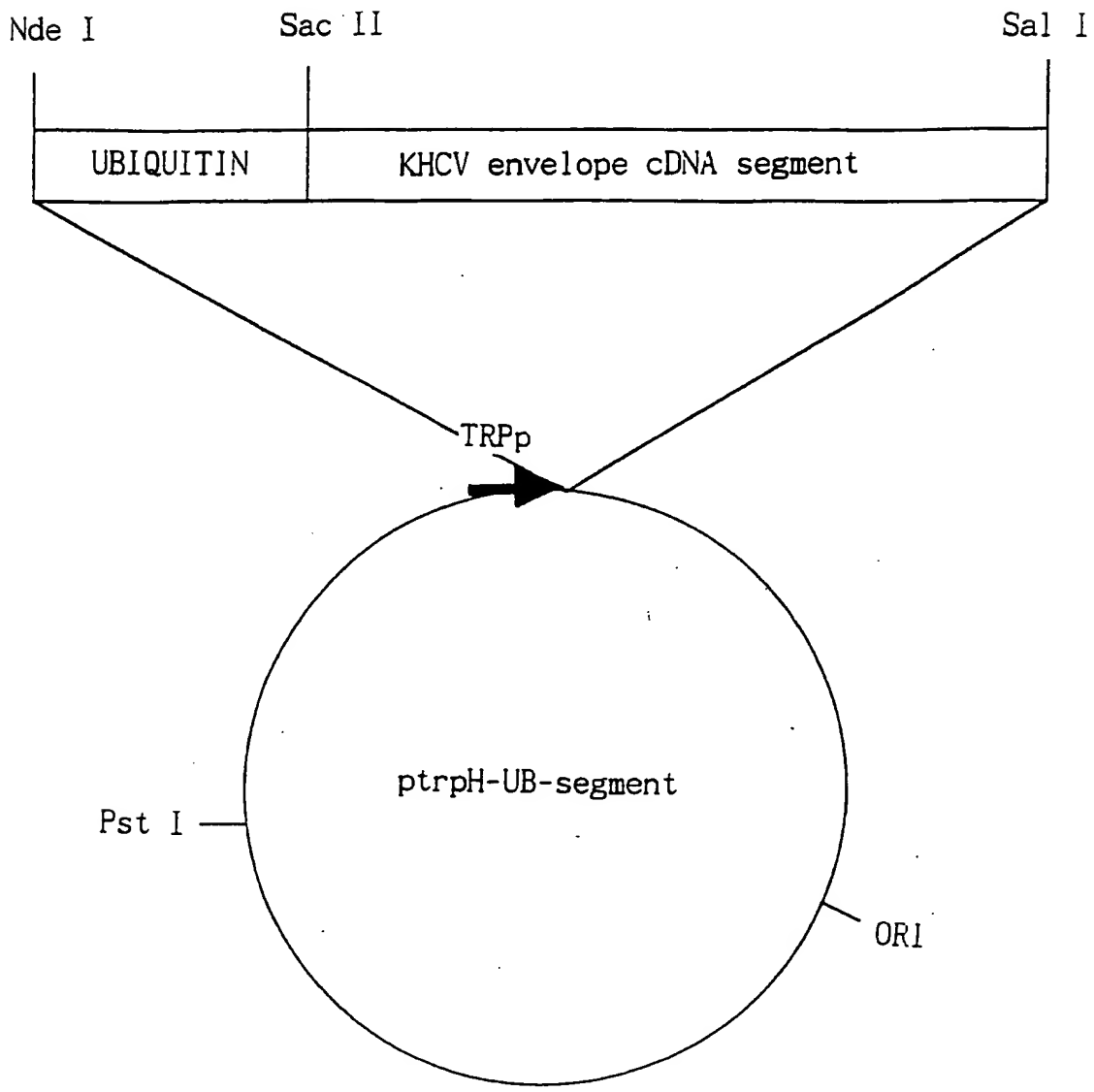


Fig. 7



SUBSTITUTE SHEET

Fig. 8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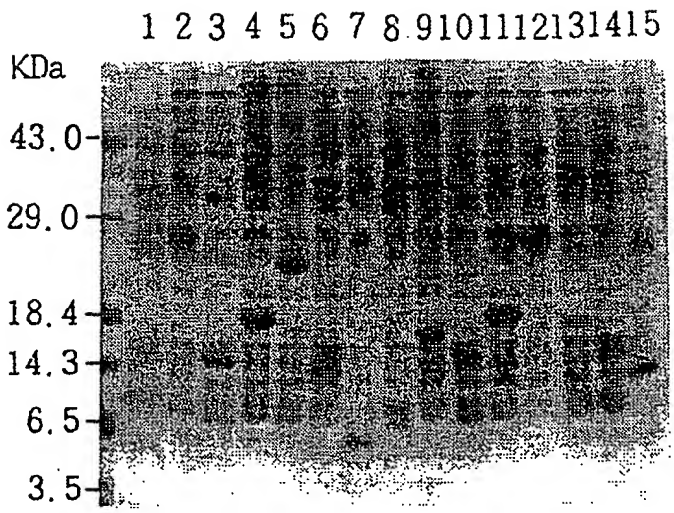
Sac II
Sal I
UBIQUITIN
KHCV envelope cDNA segment
TRPp
ptrpH-UB-segment
Pst I
ORI

Fig. 9

A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KDa
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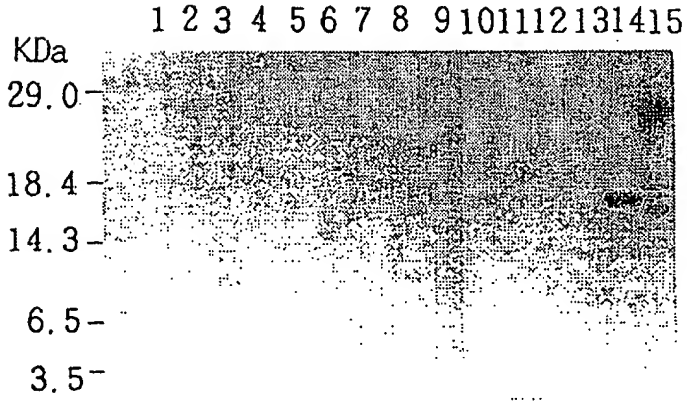
29.0
18.4
14.3
6.5
3.5

B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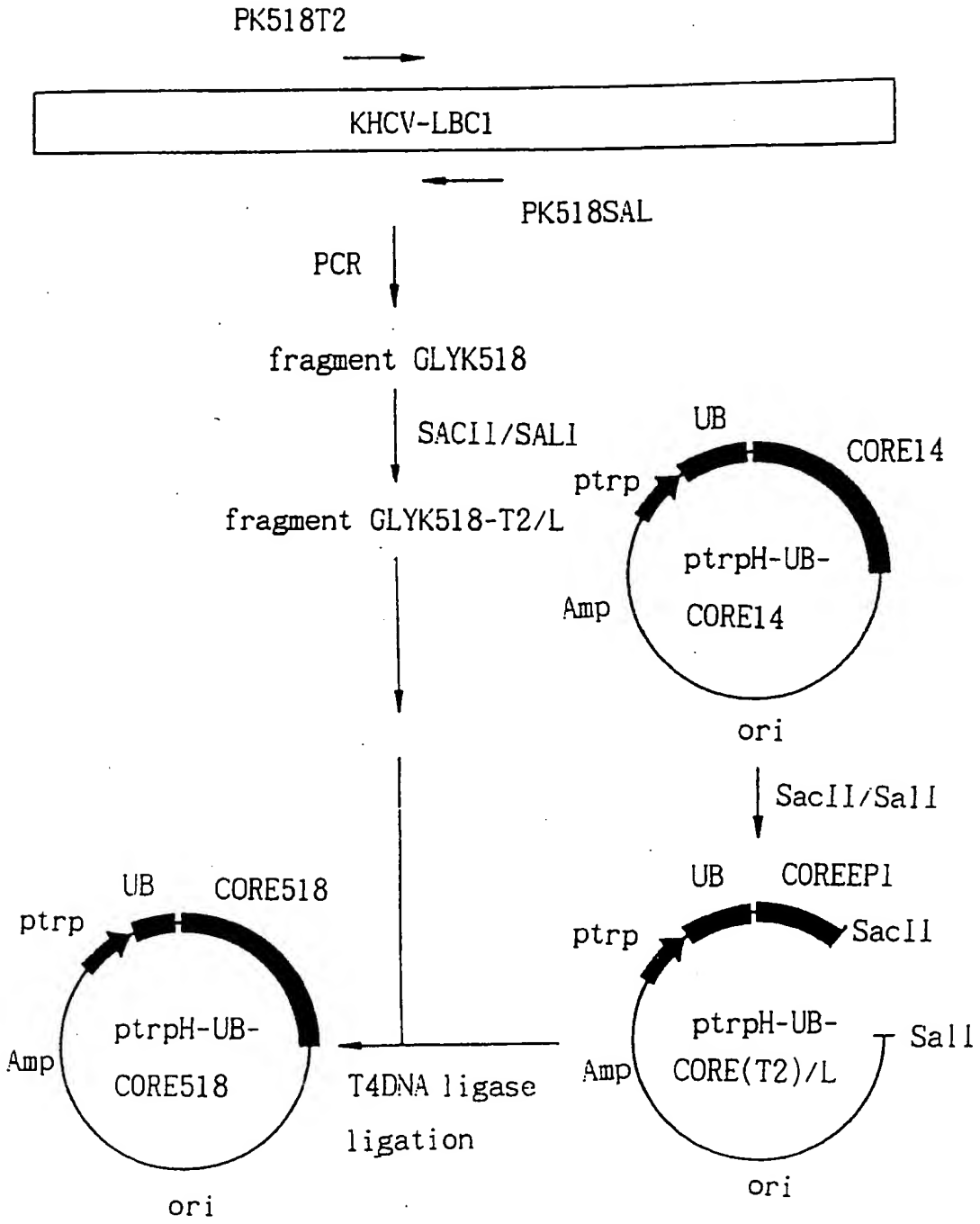
KDa
29.0
18.4
14.3
6.5
3.5

Fig. 10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KHCV-LBC1
PK518SAL
PCR
fragment GLYK518
SACII/SALI
fragment GLYK518-T2/L
UB
CORE14
ptrp
ptrpH-UB-
CORE14
Amp
ori
SacII/SalI
UB
COREEP1
ptrp
SacII
ptrpH-UB-
CORE(T2)/L
Amp
SalI
ori
UB
CORE518
ptrp
ptrpH-UB-
CORE518
Amp
ori
T4DNA ligase
ligation

Fig. 11

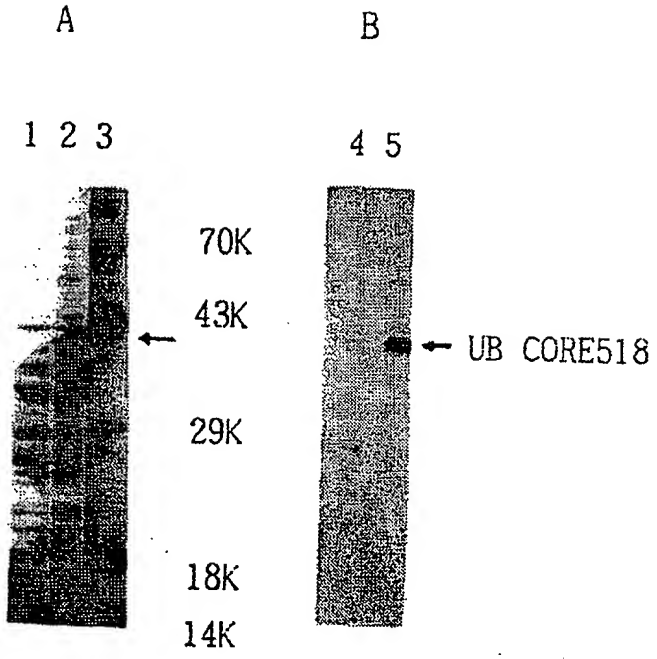


Fig. 12

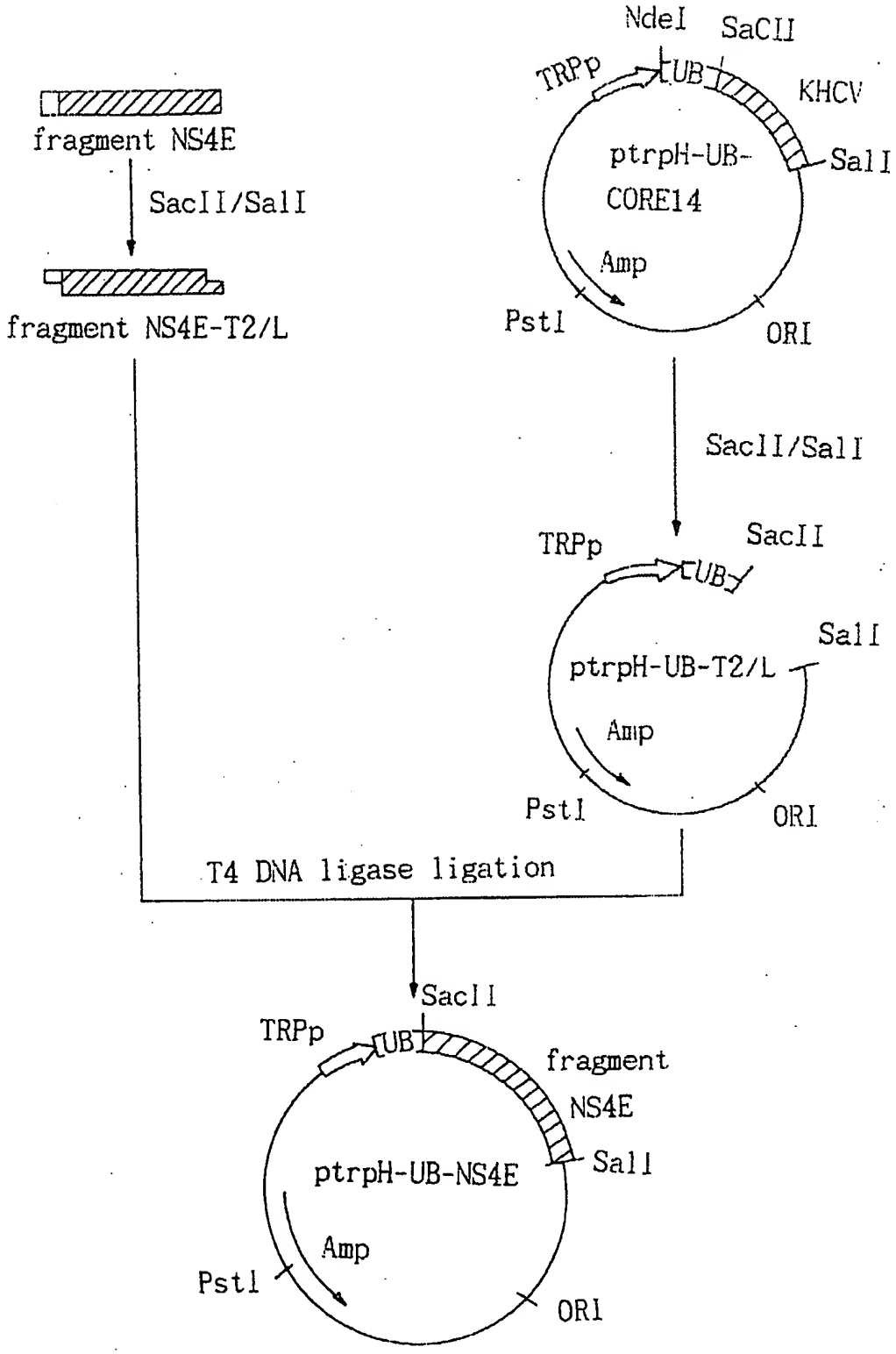


Fig. 13

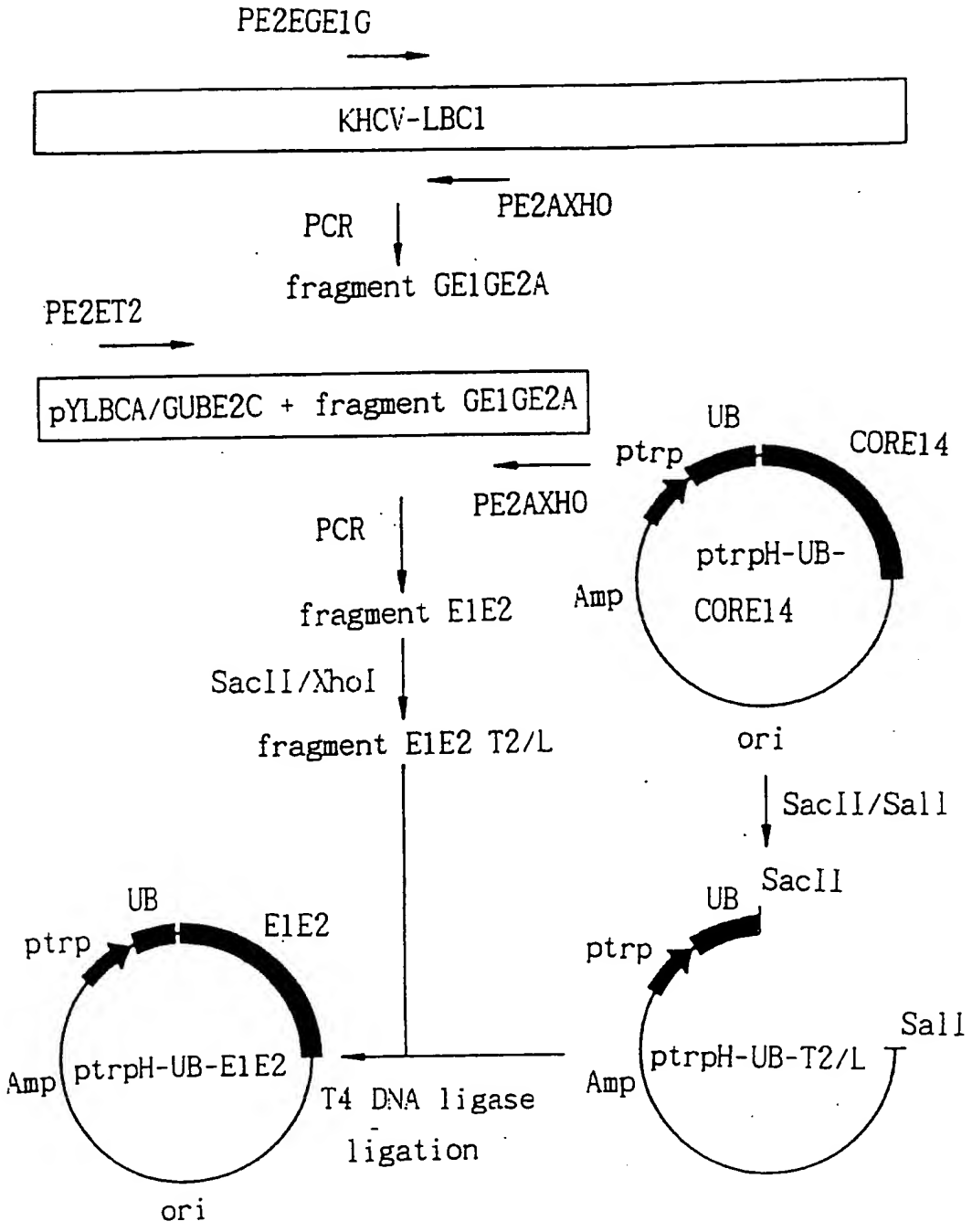


Fig. 14

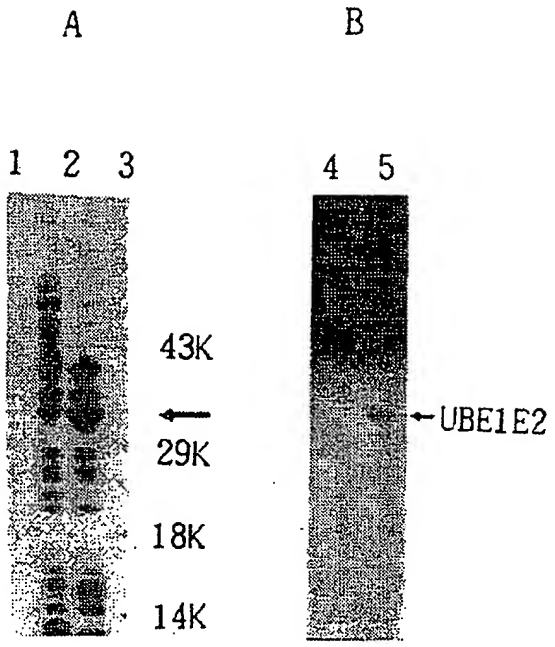
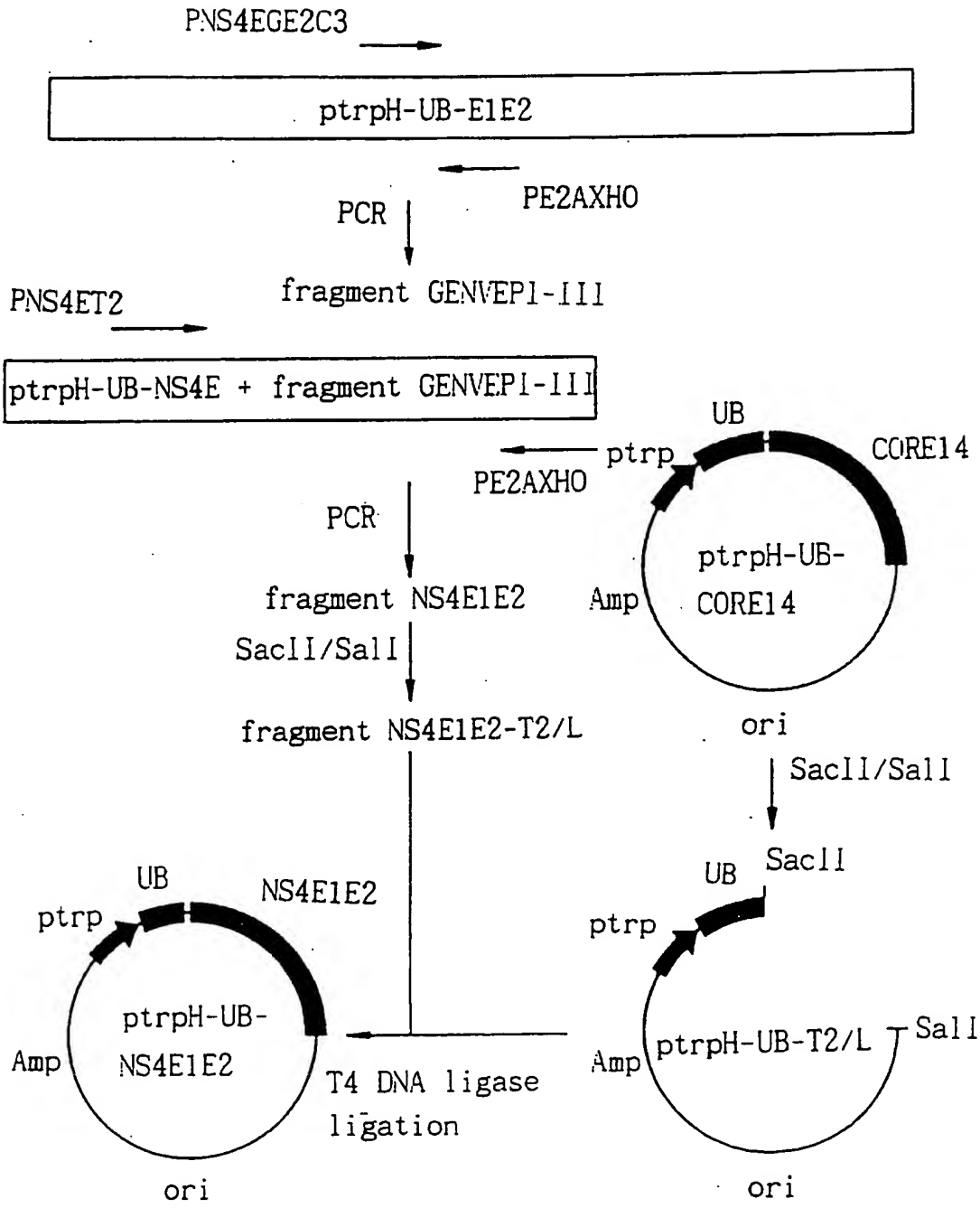


Fig. 15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ptrpH-UB-E1E2
PE2AXHO
PCR
fragment GENVEP1-III
PNS4ET2
ptrpH-UB-NS4E + fragment GENVEP1-III
PE2AXHO
PCR
fragment NS4E1E2
SacII/SalI
fragment NS4E1E2-T2/L
UB
ptrp
CORE14
ptrpH-UB-
CORE14
Amp
ori
SacII/SalI
SacII
UB
ptrp
ptrpH-UB-T2/L
SalI
Amp
ori
UB
ptrp
NS4E1E2
ptrpH-UB-
NS4E1E2
Amp
ori
T4 DNA ligase
ligation

Fig. 16

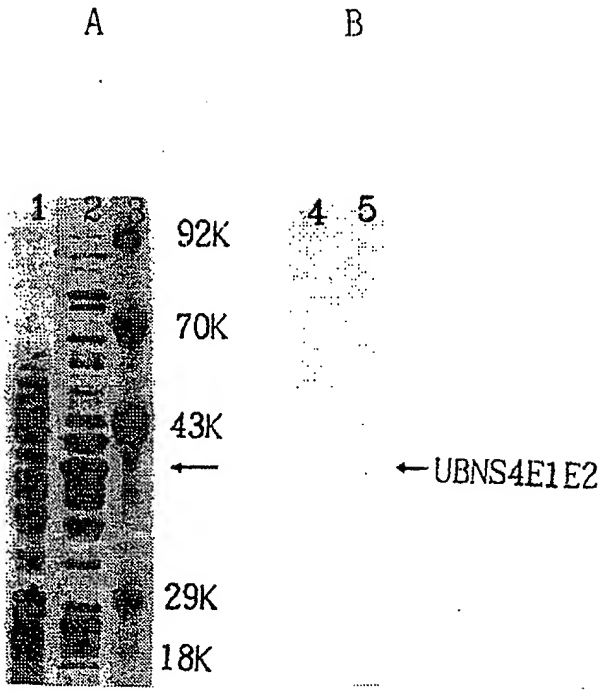
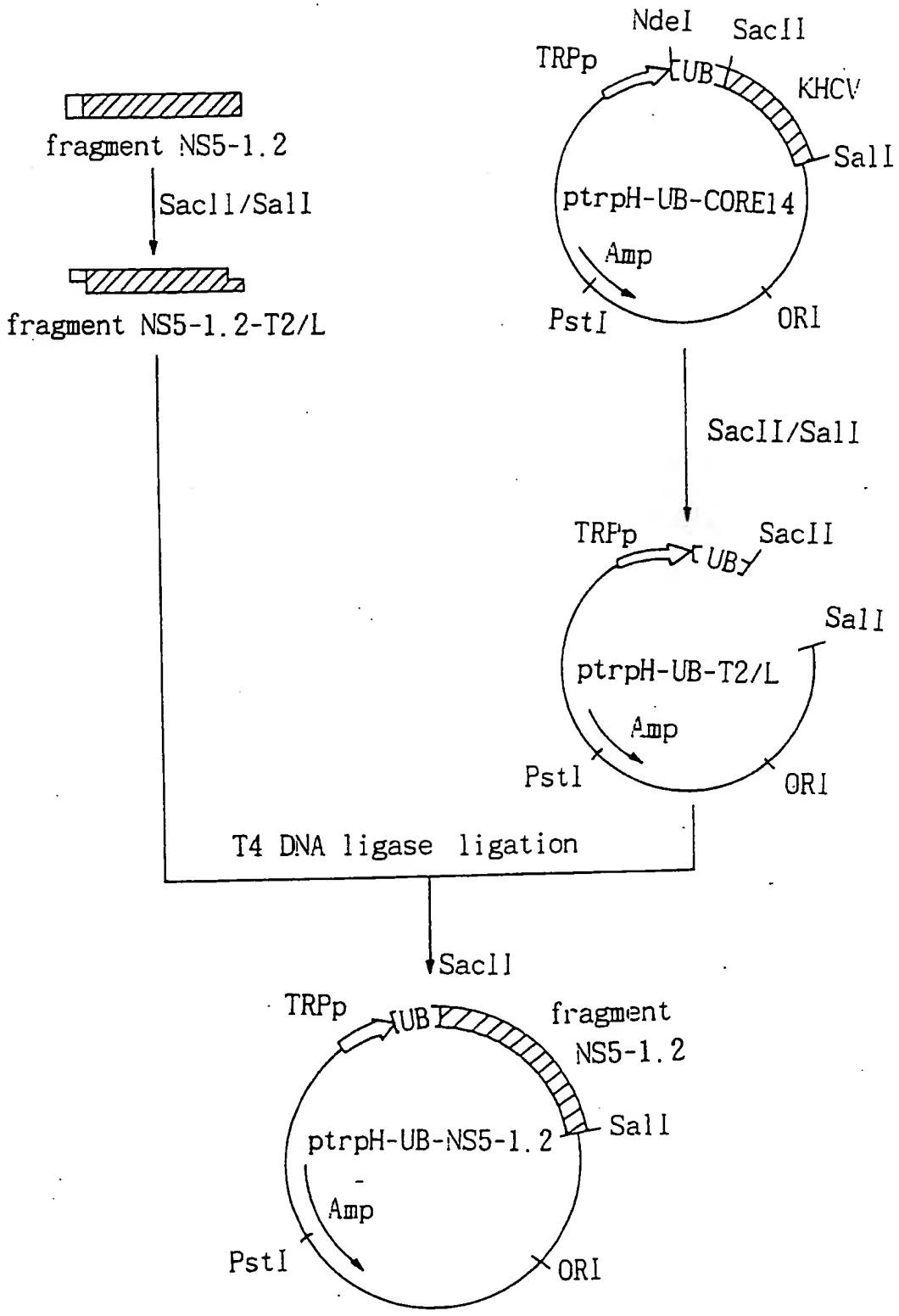


Fig. 17



SUBSTITUTE SHEET

Fig. 18

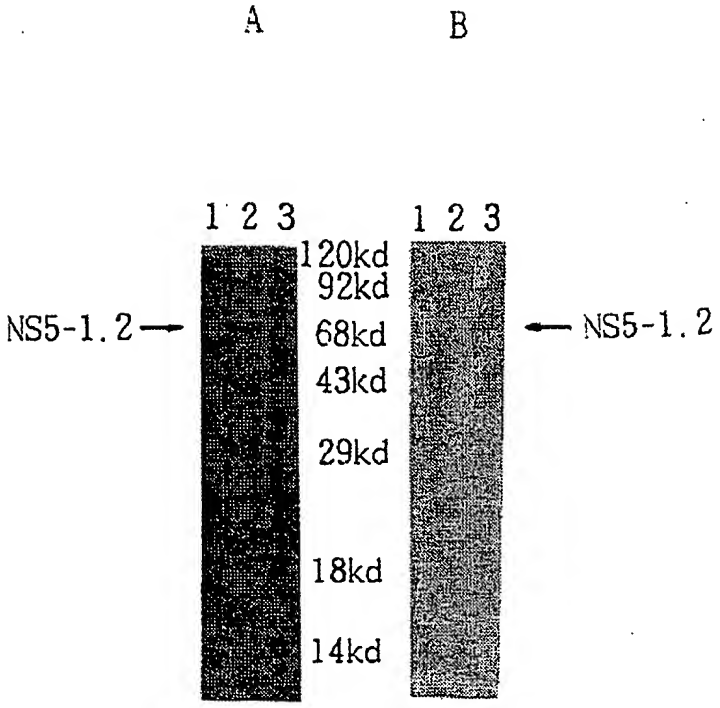
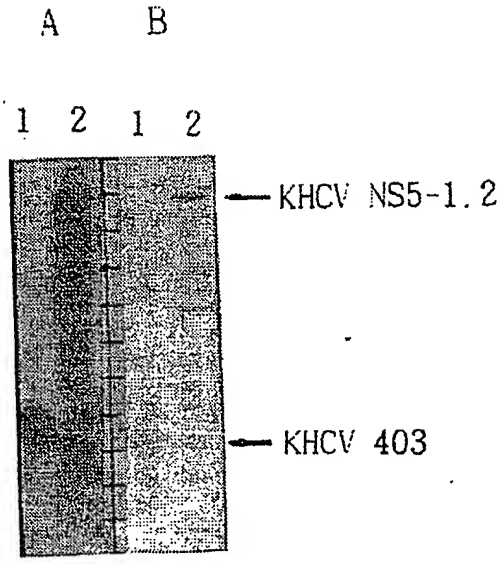


Fig. 19



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 94/00040

A. CLASSIFICATION OF SUBJECT MATTER

IPC[5]: C 07 K 13/00; C 12 N 15/51, 15/74, 1/21; C 12 Q 1/70; G 01 N 33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[5]: C 07 K; C 12 N; C 12 Q; G 01 N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AT

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0 521 318 (LUCKY LTD.) 07 January 1993 (07.01.93), claims 1,18,19,25,29,37,38,41,42,57,58.	1-21
A	CHEMICAL ABSTRACTS, Vol.116, Nr.21, issued 1992, May 25, Columbus, Ohio, USA J.E.Chang et al. "Nucleotide sequence of a cDNA fragment of the hepatitis C virus genome derived directly from Korean healthy carriers" column 2, abstract nr. 208 921 x, &.Mol.Cells 1991, 1(4), 507-10, (Eng.).	7-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1994 (14.07.94)	10 August 1994 (10.08.94)
Name and mailing address of the ISA/ AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Scharf e.h. Telephone No. 1/5337058/29

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 94/00040

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 20 and 21 are directed to diagnostic methods, which is subject matter the International Searching Authority is not required to search under Article 17(2)(a)(i) and Rule 39(iv), the search has been carried out and based on the alleged effects of the compounds.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 94/00040

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2 521318	07-01-93	AU A1 19028/92	12-01-93
		CA AA 2111108	23-12-92
		CN A 1068335	27-01-93
		EP A3 521318	28-04-93
		GB A0 9324607	23-02-94
		GB A1 2272443	18-05-94
		WO A1 9222655	23-12-92